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Doctoral Program in Biomedicine and Biochemistry

CHARACTERIZATION OF A PROFILE OF EPIGENETIC ALTERATIONS INVOLVED IN THE AETIOPATHOGENESIS OF ENDOMETRIOSIS. VALIDATION OF MOLECULAR BIOMARKERS FOR DIAGNOSIS AND PROGNOSIS OF ENDOMETRIOSIS.

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València, October 2017

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CERTIFIES:

That the research work entitled "**CHARACTERIZATION OF A PROFILE OF EPIGENETIC ALTERATIONS INVOLVED IN THE AETIOPATHOGENESIS OF ENDOMETRIOSIS. VALIDATION OF MOLECULAR BIOMARKERS FOR DIAGNOSIS AND PROGNOSIS OF ENDOMETRIOSIS**" has been entirely performed by Mr. Josep Marí Alexandre under her direction. This report is concluded and meets all the requirements for its presentation and defense as a DOCTORAL THESIS before a court.

And for that to happen for the appropriate effects, I sign the present certification in València, October the 19th, 2017.

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Dr. Juan Sandoval del Amor

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Abbreviations and acronyms

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A

ACTB: β-actin gene
Amp.: Ampicillin
ANOVA: Analysis of variance
ATCC: American Type Culture Collection
ATP: Adenosine triphosphate
AU: Arbitrary units

B

BCA: Bicinchoninic acid
bp: Base pair
BSA: Bovine serum albumin
bs-DNA: Bisulphite-converted DNA

C

C: Cytosine
ºC: Celsius degrees
CA: Cancer antigen
cDNA: Complementary DNA
CGI: CpG island
CNT: Control endometrium or endometrium from control women
CPF: Control peritoneal fluid or peritoneal fluid from control women
CpG: Dinucleotide of cytosine and guanine (5' → 3' direction). "p" stands for the phosphate bound
CRISPR: Clustered regularly interspaced short palindromic repeats

D

Da: Dalton
dCas9: Deficient CRISPR-associated nuclease
ddH_2O: Bidistillated water
DDT: Dichlorodiphenyltrichloroethane
DGCR8: Di George Syndrome Critical Region 8 protein
DIE: Deep Infiltrating Endometriosis
DMR: Differentially methylated region
DMEM: Dulbecco's modified Eagle's medium
DMSO: Dimethyl sulphoxide
DNA: Deoxyrribonucleic acid
Dnmt: DNA methyltransferase protein
dsDNA: Double-stranded DNA

E

e.g.: exempli gratia
ECM: Extracellular matrix
EDTA: Ethylenediaminetetraacetic acid
EFI: Endometriosis Fertility Index
EGTA: Ethylene glycol-bis(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid
ELISA: Enzyme-linked immunosorbent assay
EMBL: European molecular biology laboratory
END: Endometriosis
EPF: Endometriotic peritoneal fluid or peritoneal fluid from patients with endometriosis
EUT: Eutopic endometrium or endometrium from patients with endometriosis

F

FBS: Fetal bovine serum
FC: Fold change

G

GB: Gene body
GFP: Green fluorescent protein

H

h: hour
hsa: Homo Sapiens
HCT-116: Human colorectal cancer cell line 116
HEK: Human embryonic kidney cell line
HELP: HpAII tiny fragment enrichment by ligation-mediated PCR
HEPES: 2-[4-(2-hydroxyethyl)piperazin-1-yl]ethanesulfonic acid

I

IDAT: Intensity data
IL: Interleukin

K

Kb: Kilobases
KCl: Potassium chloride

L

LM-PCR: Ligation-mediated PCR
LNA: Locked nucleic acid

M

MeDIP: Methylated DNA immunoprecipitation
mg: Milligram
MIF: Macrophage inhibitory factor
min: Minute
MIRA: Methylation-based inference of regulatory activity
miRNA: microRNA
mL: Millilitre
mM: millimolar
mm^3: Cubic millimetre
MnCl2: Manganese chloride
mRNA: Messenger RNA

N

Na_2MoO_4: Sodium Molybdate
Na_2VO_4: Sodium vanadate
NaCl: Sodium chloride
ncRNA: Non-coding RNA
NET: Neutrophil extracellular traps
ng: Nanogram
NGS: Next generation sequencing
nm: Nanometre
nM; nanomolar
nt Nucleotide

O

OMA: Ovarian endometrioma
O/N: Overnight

P

P: Promoter
PAI: Plasminogen activator inhibitor protein
PAM: Protospacer adjacent motif
PBS: Phosphate Buffered Saline
PCA: Principal Component Analysis
PCR: Polymerase Chain Reaction
PDGF: Platelet-derived growth factor protein
PF: Peritoneal fluid
pg: Picogram
pH: Potential of hydrogen
PI: Peritoneal implant
***PLAU*:** uPA gene
Pre-miRNA Precursor miRNA
Pri-miRNA Primary miRNA

Q

qRT-PCR: Quantitative real-time polymerase chain reaction

R

rASRM: Revised American society for reproductive medicine
RISC: RNA-induced silencing complex
RNA: Ribonucleic acid
ROS: Reactive oxygen species
rpm: Revolutions per minute
RT: Room temperature or retrotranscription (depending on the context)
RVN: Rectovaginal Nodule

S

SCR: Scrambled
SD: Standard deviation
SEM: Standard error of mean
SERPINE1: Human Serpin Family E Member 1 or PAI-1 gene
sgRNA: Single guide RNA
siRNA: Short-interfering RNA

T

THBS1: Human Thrombospondin-1 gene
Tm Melting temperature
TMB: Tetramethylbenzidine
TPE: Tris-phosphate-EDTA buffer
TRBP: Trans-activation response RNA-binding protein
TSG: Tumour suppressor gene
TSP-1: Thrombospondin-1 protein

U

U: Unite or uracil (depending on the context)
UCSC: University of California, Santa Cruz
uPA: Urokinase plasminogen activator protein
UTR: Untranslated region
UV: Ultraviolet

V

V: Volt
VAZF3a3L: Fusion gene encoding the Dnmt3a-C, the C-terminal domain of the human Dnmt3L and an artificial zinc-finger protein targeting the *VEGFA* promoter.
VEGFA: Human Vascular Endothelial Growth Factor A gene
VEGF-A: Vascular Endothelial Growth Factor A protein
VEGFR: VEGF receptor protein
Vf: Final volume

X

X-Gal: 5-Bromo-4-Chloro-3-Indolyl -D-Galactopyranoside

Z

ZFP: Zinc finger protein

Abbreviations starting with Greek symbols

λ: Wavelength
µg: Microgram
µL: Microlitre
µm: Micrometre
ØPF: absence of PF exposure

Als meus pares, per permetre'm el viatge.

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I. Introduction

I. Introduction

1. Endometriosis

1.1. Definition and demographic data

Endometriosis is an oestrogen-dependent inflammatory disorder defined by the presence of endometrial-like tissue in ectopic locations, which limits the quality of life of affected women (Giudice and Kao, 2004; Giudice, 2010; Tamaresis et al, 2014). This pathology affects 10% of reproductive age women from all ethnic and social groups, although the prevalence in those patients experiencing pain, infertility or both is as high as 35%-50% (Burney and Giudice, 2012), being the estimated prevalence of this condition around 176 million worldwide.

Endometriosis is associated with an average diagnostic delay of 7 years (Nnoaham et al, 2011), being the mean age at diagnose 32.5-36.4 years, depending on the study population (Nnoaham et al, 2011; Fuldeore et al, 2015). This could be partially explained by the fact that the gold standard for diagnosis of endometriosis requires direct visualization of lesions at surgery followed by histological confirmation of endometrial glands and stroma in biopsies of suspected lesions. Additionally, other factors that contribute to the diagnostic delay are treatment of pain with oral contraceptives or nonsteroidal anti-inflammatory drugs and the assumption of dysmenorrhea as a normal event (Fassbender et al, 2015).

Endometriosis impairs health-related quality of life especially in the domains of pain, psychological and social functioning, which results in an important loss of productivity (Fourquet et al, 2011; Nnoaham et al, 2011). Despite of being a benign disease, endometriosis deeply affects the quality of life of affected women, also implying a highlighting economic cost. The World Endometriosis Research Foundation (WERF) performed the EndoCost study, intended to estimate the costs related to endometriosis care by means of a prospective, multi-center, questionnaire-based survey in ambulatory care in 12 centers from 10 countries, enrolling 909 women. An average total annual cost per woman of 9,579€ was calculated. Moreover, this amount increased in parallel with severity of endometriosis and years of delayed diagnosis (Simoens et al, 2012). Of them, 3,133€ were the average total annual direct health costs. Interestingly, this amount is similar to the estimated annual health care costs for diabetes mellitus, Crohn's disease and rheumatoid arthritis in European countries, which points to the considerable economic burden of this condition for national health systems (Simoens et al, 2012).

1.2. Types of endometriotic lesions

Endometriosis is defined by the presence of endometrial-like tissue in ectopic locations. The most frequent involvement is in the peritoneum (superficial and deep endometriotic implants) and ovaries (endometrioma or endometriotic cysts), although cases of pulmonary (Huang et al, 2013) and cerebral endometriosis (Thibodeau et al, 1987) have also been documented.

From a clinical point of view, there are three main distinct lesions depending on their location: peritoneal implants (PI, on the surface of the peritoneum), ovarian endometriomas (OMAs, in the ovary), and rectovaginal nodules (RVN; in the Pouch of Douglas and rectovaginal septum). All of them will be described in detail hereafter. Even though it remains uncertain whether these three types are variants of the same pathologic process or are caused by different mechanisms (Nisolle and Donnez, 1997; Bulun, 2009), anyhow it seems clear that their specific clinical features warrant an individualized analysis of their biological behavior.

From a histological point of view, all three types of lesions share common features, as the presence of stromal and epithelial endometrial cells, chronic bleeding and signs of inflammation (Bulun, 2009).

a) Ovarian endometrioma

OMAs are cysts lined by endometrioid glands histologically and functionally similar to EUT that are generated by an invagination process of implants in the ovarian cortex (**Fig. 1**). These endometriotic lesions are also called "chocolate cysts" because of the presence of an internal fluid generated by the accumulation of menstrual debris as consequence of the shedding of the active implants inside the cyst (Sánchez et al, 2014). The most active OMAs present areas of endometrial lining, with abundant accumulations of hemosiderophages and mild parietal fibrosis. When cysts are inactive or in regression, intense cicatricial hyaline fibrosis, hemosiderophages and absence of endometrial tissue are objectified (Scurry J et al, 2001).

With regards to fertility, ovarian endometriosis has a deleterious effect, which could be explained by several mechanisms. Firstly, OMA contains free iron, reactive oxygen species (ROS), proteolytic enzymes and inflammatory molecules in concentrations from tens to hundreds of times higher than those present in peripheral blood or in other types of benign cysts, potentially damaging the surrounding healthy ovarian tissue (Sánchez et al, 2014). Secondly, there is a reduction in both embryo and oocyte quality as consequence of the oocyte development in an unfavorable microenvironment (Stilley et al, 2012). This fact is reinforced by the observation of a reduction in donor oocyte implantation rates with endometriosis in recipients without the disease (Navarro et al, 2003; Stilley et al, 2012). Thirdly, ovarian reserve is negatively affected following surgical excision of these cysts (Garcia-Velasco and Somigliana, 2009). And finally, endometriosis may contribute to sterility as a result of the alteration of endometrium and the decrease in endometrial receptivity as a consequence of a chronic inflammation produced by the disease, but this is far from the scope of this work.

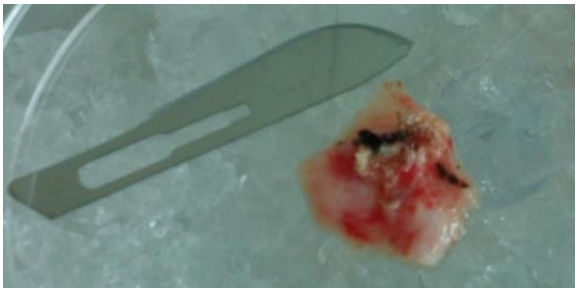


Figure 1: Biopsy of ovarian endometrioma being processed in our laboratory.

Of note, ovarian endometriosis has been classified as a tumour-like condition by the World Health Organization Histologic Classification of Ovarian Tumours (Scully, 1987). Almost a hundred years ago, Sampson published the article "Endometrial carcinoma of the ovary, arising in endometrial tissue in that organ", becoming the first to report a case of suspected malignant transformation in endometriosis (Sampson, 1925). Though several studies have focused on the relationship between endometriosis and gynaecological cancers, especially the endometrioid and clear cell subtypes of ovarian cancer (Melin et al, 2006; Munksgaard et al, 2011), recent literature on this issue points that existing data is not enough to establish a doubtless causality. This is, there is a lack of studies demonstrating clear temporal changes from benign lesions to malignant ones also establishing a clear phylogenetic relationship between ovarian cancer and its adjacent endometriotic lesions. Nevertheless, the estimated prevalence of 1% of patients with endometriosis developing ovarian cancer seems to be related to the hazardous co-existence of two unrelated conditions in a reduced anatomical space as the pelvic peritoneum, rather than a malignant transformation of OMAs (Guo, 2015).

b) Peritoneal implant

According to their macroscopic appearance, superficial PIs can be categorized in red, black and white implants (**Fig. 2**) (Donnez J et al, 2003; Gilabert-Estellés J, 2011):

- **Red peritoneal implants** may appear in different forms: petechial, flame-like and vesicular lesions. They all present a great vascularization and show great similarity with EUT (in terms of the state of glandular proliferation and the stromal vascularisation), thus reinforcing Sampson's theory. This fact suggests that red lesions constitute the initial stage of peritoneal implantation (also called active or functional when secretory changes happen).

- **Black peritoneal implants** could appear as black-blue, powder-burn and brown lesions. Black implants might be the consequence of the inflammatory reaction caused by the implantation of the migrated tissue, which would ultimately result in encapsulation of the lesion and reduction of stromal vascularisation. Additionally, blood vessels show more thickened walls, the lesion being deeper, favouring inflammation, hemorrhage and hemosiderin deposits that are phagocytosed by macrophages, leading later to fibrosis. Therefore, these lesions are considered intermediate, non-active advanced endometriosis.

- In cases where inflammation and subsequent fibrosis completely devascularize the endometriotic focus, **white collagen plaques** appear, remaining in the ectopic lesion. This is the origin of the different forms (namely fibrosis, white plaques and peritoneal defects) of latent, inactive PIs (Nisolle and Donnez, 1997).

To complete a panoramic view of superficial peritoneal lesions, another classification considers the frequency in which they are detected by laparoscopy to categorize them as typical (black powder-burn lesions) or atypical implants (the rest of the aforementioned).

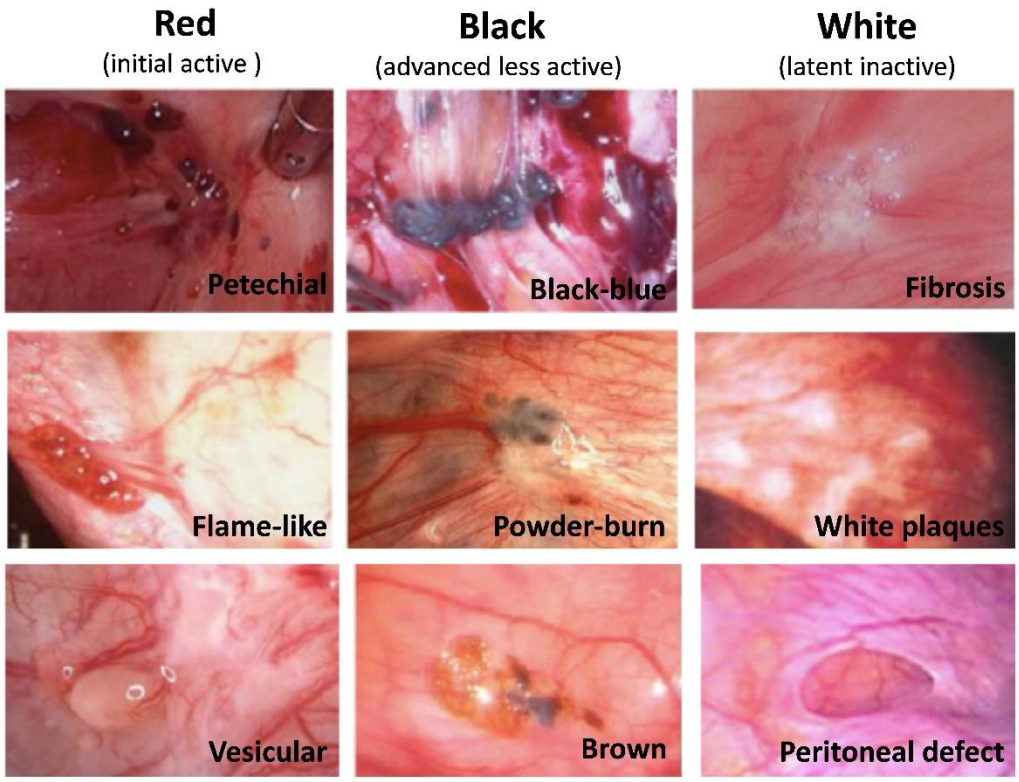


Figure 2. Different types of superficial peritoneal endometriotic implants (Modified from Gilabert-Estellés, 2011).

c) Rectovaginal nodules

Deep endometriosis implants represent the so called deep infiltrating endometriosis (DIE), defined as a particular form of endometriosis with lesions penetrating >5 mm under the peritoneal surface (Koninckx and Martin, 1994). DIE implants are located in specific locations, primarily the posterior area, involving uterosacral ligaments, torus uterinus, the posterior vaginal wall and the anterior rectal wall (Chapron et al, 2003). Of special interest are the endometriotic lesions termed RVN, which will be described thereafter.

RVN are fibrotic masses comprising endometriotic, adipose and fibromuscular tissues originated as the result of a process of infiltration of the space between the rectum and the vagina, rendering a tissue with few vessels and endometrial glands and a high fibrous component and collagen (Gilabert-Estellés et al, 2012). These lesions are considered very active, usually infiltrate the structures in the vicinity (**Fig. 3**) and are strongly associated with pelvic pain symptoms (Chapron et al, 2003).

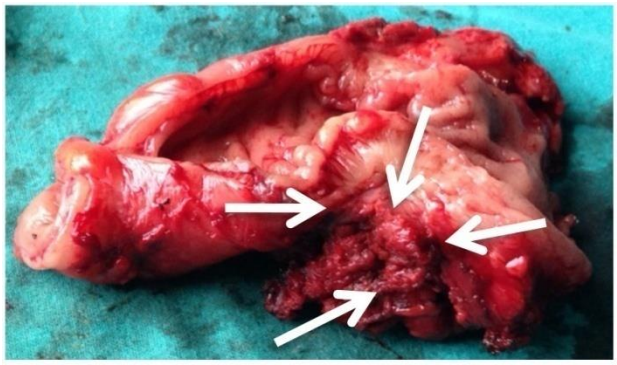


Figure 3. Rectovaginal endometriosis involving the rectum after bowel resection. White arrows delimitate the RVN. Courtesy of Dr. Javier García-Oms.

1.3. Endometriosis classification system

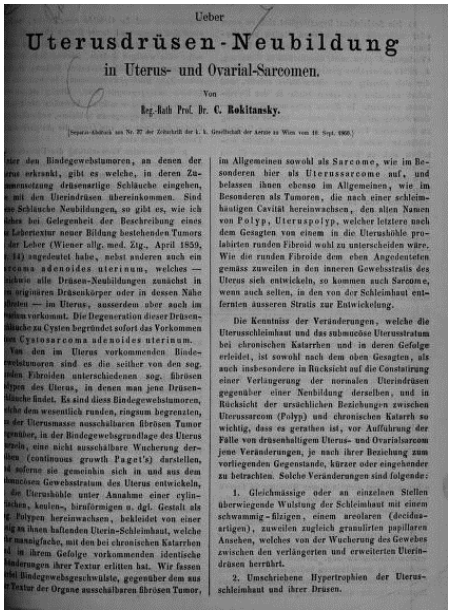
Endometriosis has a classification system validated in four stages, which was established by the American Society of Reproductive Medicine in its revised version in 1997 (rASRM, 1997). Although this classification system failed to predict probability of pregnancy following treatment, it is still in use due to its utility in providing a mean of clearly documenting extent and location of disease.

The rASRM classification is based on a punctuation system considering the presence of endometriotic lesions in the peritoneum and ovary, obliteration in the pouch of Douglas, and tubal and ovarian adhesions, assessed during surgery. According to the obtained score, a patient can be diagnosed of endometriosis in stages I to IV, where stage I represents minimal disease; stage II mild disease; stage III moderate disease and stage IV represents severe endometriosis. However, this classification failed predicting both the level of pain and the probability of pregnancy following treatment (rASRM, 1997).

To overcome the limitation related to spontaneous pregnancy prediction after a surgery for endometriosis, the Endometriosis Fertility Index (EFI) score has been developed (Adamson and Pasta, 2010). EFI score is also based on a punctuation system which takes into account both surgical factors assessed during surgery (representing half of the points and involving fallopian tubes, fimbriae and ovaries) and historical factors of the woman (involving age, years of infertility and whether a prior pregnancy occurred). The EFI score ranges from 0–10, with 0 representing the poorest prognosis and 10 the best prognosis. Importantly, the EFI score has been validated in a French (Boujenah J et al, 2015), Italian (Garavaglia et al, 2015) and Belgian (Tomassetti et al, 2013) cohorts, but not yet in a Spanish one.

1.4. Aetiopathogenesis of endometriosis

Despite extensive research endeavours, a unifying theory regarding the exact aetiopathogenic mechanism of this high prevalent and incapacitating condition is still lacking (Giudice, 2010). Conversely, different theories have emerged in an attempt to explain the different clinical presentations of the disease (Vinatier et al, 2001; Giudice and Kao, 2004; Jiang and Wu, 2012) since the Austrian pathologist Karl von Rokitansky first described it at the 19th century in his publication "About uterine gland formation in uterine and ovarian Sarcoma" (Rokitansky, 1860) (**Fig. 4**).



Ueber

Uterusdrüsen-Neubildung

in Uterus- und Ovarial-Sarcomen.

Von

Reg.-Rath Prof. Dr. C. Rokitansky.

[Separat-Abdruck aus Nr. 37 der Zeitschrift der k. k. Gesellschaft der Aerzte zu Wien vom 16. Sept. 1860.]

Figure 4. First page of the paper entitled "About uterine gland formation in uterine and ovarian Sarcoma", in which von Rokitansky first reports endometriotic lesions (In the Internet Archive, recovered in October, 2nd, 2017 at https://archive.org/details/b22326558).

Theories can be grouped into those proposing a uterine origin of implants and those proposing an extrauterine origin of endometriotic lesions:

1.4.1. Theories regarding a uterine origin of endometriosis

Two theories account for those proposing a uterine origin of endometriotic lesions, namely the theory of retrograde menstruation and the theory of benign metastasis (both hematogenous and lymphatic).

Initially proposed by the American gynaecologist J.A. Sampson in 1927 (Sampson, 1927), the **theory of the retrograde menstruation and implantation** is the most widely accepted. According to Sampson's proposal, endometrium shed during menstruation is capable of reaching the abdominal cavity by retrograde flow through the fallopian tubes, then being able to implant and proliferate. Albeit it has been documented that retrograde menstruation occurs in 90% of healthy women in reproductive age with patent fallopian tubes (Halme et al, 1984), the fact that only a small percentage develops the disease suggests that there must be additional mechanisms that allow the migrated tissue to implant and survive (Konninckx et al, 1999).

This theory is supported by numerous studies and findings, as the high prevalence of endometriosis in women with anatomical impediments to the antegrade menstruation (e.g. because of congenital outflow obstruction (Sanfilippo et al, 1986), uterine septum (Nawroth et al, 2006), cervical stenosis (Barbieri 1998) and in baboon models with iatrogenic obstruction of the outflow (D'Hooghe et al, 1994). Additionally, the finding of a left lateral predisposition of peritoneal endometriosis and OMAs support retrograde menstruation theory, provided the decreased fluid movement in the left side because of the presence of sigmoid colon (Al-Fozan et al, 2003; Bricou et al, 2009).

The closely related **theory of benign metastasis** proposes that endometrial cells reach the peritoneal cavity through lymphatic or hematogenous dissemination, then establishing endometriotic lesions. In addition to having been demonstrated in baboons (Hey-Cunningham et al, 2011), this could provide an explanation for the documented presence of endometriosis in lung (Huang et al, 2013) and brain (Thibodeau et al, 1987).

1.4.2. Theories regarding a non-uterine origin of endometriosis

Four theories account for those proposing a non-uterine origin of endometriosis, namely **coelomic metaplasia theory** (Iwanoff, 1898), **induction theory** (Levander and Normann, 1955; Merrill, 1966), **embryonic Müllerian rests theory** and the recently proposed **extrauterine bone-marrow progenitor theory**.

- Both *coelomic metaplasia* and *induction theory* propose that endometriotic lesions are the result of the transformation of normal peritoneum under the influence of appropriate stimulus (e.g. endocrine disruptors for coelomic metaplasia and endogenous hormonal or immunologic factors for induction theory). In a recent congress (I Congress of the Society for Endometriosis and Uterine Disorders; Paris, 2015), Dr. Yamagata presented a brilliant oral communication, his results providing a further piece of support for a peritoneal origin of endometriotic lesions. He and co-workers analysed the DNA methylome (see Section 3.1.2. for detailed explanation) of endometrial stromal cells from OMAs, and tissues from OMAs, normal ovarian cortex, PIs, normal peritoneum and EUT. Cluster analyses determined that samples from OMAs and PIs were more similar to their respective healthy tissues than to EUT tissues. Based on results coming from cancer studies, proving that DNA methylome in metastasis are more similar to methylome of primary tumour than those of the surrounding tissue (Hao et al, 2017), authors concluded that OMAs lesions develop from ovary and PIs from healthy peritoneum, rather than from EUT tissues (Yamagata el al, 2015).

- The idea that oestrogen could also induce the formation of endometriotic lesions has also developed by the *theory of embryonic Müllerian rests*. In contrast to the induction theory, the former proposes that residual cells from embryonic Müllerian duct migration develop into endometriotic lesions under the influence of oestrogen beginning at puberty (Russell, 1899).

- Finally, the more recent proposed *theory of bone marrow progenitors* suggests that extrauterine bone marrow mesenchymal stem progenitors and endothelial progenitors may differentiate into endometriotic tissue (Sasson and Taylor, 2008).

These theories are backed by clinical findings of histologically proven endometriotic tissue in patients without menstrual endometrium (e.g. women with Rokitansky-Kuster-Hauser syndrome) and men with prostate cancer undergoing treatment with high-dose of estrogens (Burney and Giudice, 2012).

1.5. Pathophysiologic mechanisms involved in endometriosis

Endometriosis is a multifactorial disease in which endometrial and peritoneal factors such as those related to angiogenesis and proteolysis may be involved (Kobayashi, 2000; Zondervan et al, 2007; Braza-Boïls et al, 2014).

1.5.1. Angiogenesis

Angiogenesis is the process of formation of new blood vessels from pre-existing ones. It occurs physiologically during embryogenesis and in adult organisms in the process of wound healing and fractures. However, in the female reproductive apparatus an exception occurs, due to the post-menstrual need of cyclical regeneration of the endometrium (Augustin, 2000).

Although until the 1970s the molecular basis of angiogenesis was largely unknown, nowadays it is accepted that angiogenesis is a complex process regulated by a delicate balance between endogenous promoters (pro-angiogenic factors) and inhibitors (anti-angiogenic factors). This balance regulating the angiogenic state of a cell or tissue has been termed the "*angiogenic switch*" (Lawler and Lawler, 2012). In recent decades, extensive studies have revealed a variety of pro-angiogenic factors and their receptors, including vascular endothelial growth factor (VEGF)-VEGFRs, Angiopoietin-Tie, Ephrin-EphRs and Delta-Notch to be the major regulators of angiogenesis in vertebrates. Importantly, VEGF and its receptors play a central role in physiological as well as pathological angiogenesis and has been postulated as the main regulator of this process (Shibuya, 2008). On the other hand, TSP-1 is considered a potent endogenous inhibitor of angiogenesis (Lawler and Lawler, 2012).

1.5.1.1. Vascular endothelial growth factor A (VEGF-A)

VEGF-A belongs to the platelet-derived growth factor (PDGF)/VEGF supergene family and is a key player both in vasculogenesis (i.e. the formation of blood vessels from progenitor cells) as well as angiogenesis. Other members of the (PDGF)/VEGF supergene family in humans are VEGF-B (displaying a low pro-angiogenic activity) and VEGF-C and VEGF-D (preferentially stimulating lymphangiogenesis) (Shibuya, 2008).

VEGFA gen is located in the chromosomic locus 6p21.1 and consists of 8 exons along approximately 14 kb (43770209 - 43786487) (https://www.ncbi.nlm.nih.gov/gene/7422). Of the seven isoforms obtained by alternative splicing of this homodimeric glycoprotein (namely VEGF-A_{206}, VEGF-A_{189}, VEGF-A_{183}, VEGF-A_{165}, VEGF-A_{148}, VEGF-A_{145} and VEGF-A_{121}), the VEGF-A_{165} isoform (46kDa) is the most important both quantitatively and qualitatively.

The VEGF receptor (VEGFR) family belong to the receptor-type tyrosine kinase (RTK) supergene family and consists of three members: VEGFR1, VEGFR2 and VEGFR3. VEGFRs have 7 immunoglobulin domains and require dimerization to activate downstream signalling pathways (Shibuya, 2013).

VEGF-A binds to both VEGFR1 and VEGFR2, but with very different features. VEGFR1 has high affinity for VEGF-A, but the binding weakly stimulates angiogenesis because of the low tyrosine kinase activity of this receptor. Oppositely, VEGF-A strongly activates the

tyrosine kinase activity of VEGFR2, although their binding is about one order of magnitude weaker than that of VEGFR1. Since VEGF-B only binds VEGFR1, its pro-angiogenic activity is rather limited. On the other hand, VEGFR3 binds VEGF-C and VEGF-D, stimulating lymphangiogenesis (or the formation of lymphatic vessels from pre-existing ones) (**Fig. 5**) (Shibuya, 2008).

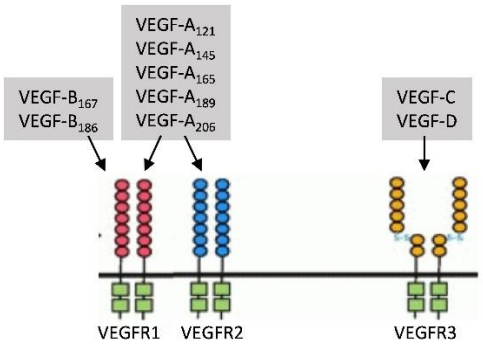


Figure 5. Members of the (PDGF)/VEGF supergene family and the receptors their preferentially bind to. VEGF: vascular endothelial growth factor; VEGFR: VEGF receptor. (Modified from In Signalling for VEGF Pathway. Garapati P.V. Recuperated on September, 29th 2017 at http://reactome.org/).

Homozygous mutants of loss of function of the two major receptors of VEGF-A, VEGFR-1-/- and VEGFR-2-/- lead to embryonic lethality, implying that both VEGF-A and its two receptors are essential for the development of the embryonic vasculature (Shalaby et al, 1995; Fong et al, 1999).

VEGFA mRNA is expressed at very distinct physiological levels in different human tissues (such as brain, thyroid glands, lung, prostate, brain and endometrium), as recently corroborated by RNA-seq (**Fig. 6**) (Fagerberg et al, 2014), and its up-regulation is a driving pathogenic event in several malignant (Guo, 2015) and benign conditions as endometriosis (Gilabert-Estellés et al, 2007; Cosín et al, 2010; Braza-Boïls et al, 2014).

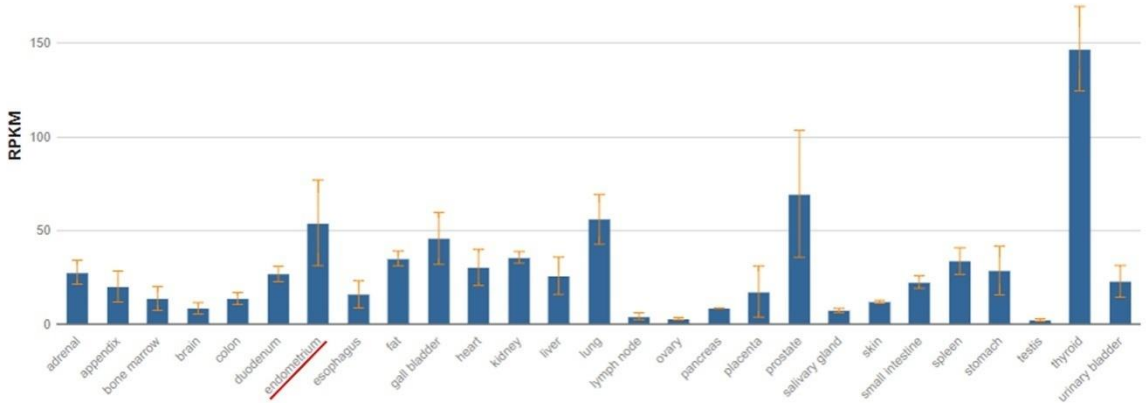


Figure 6. *VEGFA* mRNA expression across human tissues. RPKM: reads per kilobase million. (Fagerberg et al, 2014).

The expression of VEGFA is tightly regulated by a high number of factors: at the genetic level, polymorphisms (Cosín et al, 2009) and amplifications (Andreozzi et al, 2014) regulate its expression. At the epigenetic level, promoter CpG methylation (Siddique et al, 2013) and miRNA-mediated translational repression (Marí-Alexandre et al, 2015) have been proposed to regulate its expression. Finally, several studies demonstrated that VEGFA

expression is up-regulated by hypoxia, estrogen, NF-κβ (Shibuya et al, 2008) and WNT/β-catenin (Zhang et al, 2015), among and other cytokines.

1.5.1.2. Thrombospondin-1 (TSP-1)

The thrombospondin (TSP) family includes five members: TSP-1, TSP-2, TSP-3, TSP-4, and TSP-5 or COMP (Cartilage Oligomeric Matrix Protein) grouped in two subfamilies: A, with trimeric structure (TSP-1 and TSP-2) and B, with pentameric structure (TSP-3, TSP-4, and TSP-5). However, they are encoded in different genes and chromosomes, the regulatory DNA and mRNA regions differ and similarities are only related to their modular domain structure. Thus, their expression profile is also different from a spatial and a temporal point of view, suggesting their functions are also different (Stenina-Adognravi, 2013).

Nevertheless, whereas the anti-angiogenic functions of TSP-1 and TSP-2 have been known for many years, the functions of TSP-3, TSP-4 and TSP-5 remain unclear. By performing these studies in the context of the whole animal, Bornstein and collaborators (Bornstein et al, 2004) concluded that differences in the functions of TSP-1 and TSP-2 are not the consequence of differences in their intrinsic properties but of their different spatial and temporal patterns of their expression.

Although initially identified in platelet alpha granules (Lawler et al, 1978), TSP-1 was later found to be produced and secreted in different cell types, such as endothelial cells, fibroblasts, smooth muscle cells, endometrial stromal cells and pneumocytes type II (Iruela-Arispe et al, 1996; Armstrong et al, 2003). TSP-1 antagonizes VEGF-A in several important ways, via inhibition of VEGF-A release from the extracellular matrix, direct interaction and prevention of VEGF-A-VEGFR2 interaction, and inhibition of VEGF signal transduction via TSP-1-CD47-VEGFR2 interaction (Lawler and Lawler, 2012).

1.5.2. Extracellular matrix remodelling

The extracellular matrix is the non-cellular component present within all tissues and organs (Frantz et al, 2010). Although initially ECM was thought to be an inert scaffold providing support for cells and mechanical strength to tissues and organs, it is currently considered a highly active 3D network of molecules that interacts with cells, thereby regulating or instructing their behaviour. The ECM affects the phenotype of all resident cells and regulates numerous cellular events such as adhesion, migration, proliferation, differentiation, survival and immune system signalling that ultimately affect development, homeostasis and tissue repair (Piccinini and Midwood, 2014).

Cell behaviour is profoundly affected by the ECM, whose synthesis and turnover must be finely balanced in order to maintain normal function and prevent disease. In the last decade, several animal models and clinical studies have revealed that miRNAs are key regulators of ECM gene expression (reviewed in Piccinini and Midwood, 2014).

1.5.2.1 The fibrinolytic system: urokinase plasminogen activator (uPA) and plasminogen activator inhibitor-1 (PAI-1)

The fibrinolytic system is essential for the maintenance of vascular homeostasis. However, its role in many other processes such as cancer and endometriosis has recently been documented, given its importance in extracellular matrix degradation (Chapin and Hajjar, 2015).

The main enzyme of the fibrinolytic system is plasmin, responsible for the degradation of fibrin in soluble products. The passage of the zymogen plasminogen to plasmin is catalyzed by two activators, the tissue type (tPA) and the urokinase type (uPA), acting through its membrane receptor (uPAR). The activity of both, in turn, is regulated by inhibitor-specific plasminogen activators (PAIs) (Zorio et al, 2008). Among PAIs, the inhibitor of plasminogen activator type 1 (PAI-1) is the most important, produced mainly by vascular endothelial cells, but also by different benign and malignant tumours and by macrophages (Bruse, 1998).

1.5.2.2. Angiogenesis and proteolysis in endometriosis

As in tumour metastases, endometriotic implants require neovascularization to proliferate, invade the ECM and establish the lesion (Laschke and Menger 2007). Previous results from our group showed that protein levels of VEGF-A were significantly elevated in EUT from patients with respect to the endometrium of women without endometriosis. Considering endometriotic lesions, PIs had levels of VEGF-A significantly increased compared to OMAs (Gilabert-Estellés et al, 2007; Cosín et al, 2010).

Rather than a pathological process, the process of angiogenesis in the endometrium is monthly required after menstruation to regenerate the functional layer of this mucosa from the basal layer. However, several authors, including our group, coincide in that there is a difference in the expression of proangiogenic (VEGF-A) (McLaren et al, 1996; Donnez et al, 1998; Fasciani et al, 2000; Gilabert-Estellés et al, 2007; Ramón et al, 2011), antiangiogenic (TSP-1) (Tan et al, 2002; Kawano et al, 2005; Gilabert-Estellés et al, 2007; Ramón et al, 2011) and proteolytic (plasminogen activator type urokinase, uPA; Metalloprotease-3, MMP-3) factors in the EUT of women with endometriosis compared to the CNT, which could favour the implantation of the tissue in ectopic locations.

ECM remodelling plays a critical important role in the establishment of endometriotic lesions. Abnormal expression of components of metalloproteinase systems at the mRNA level has been reported in both the endometrium and endometriotic tissue of women affected by endometriosis (Ramón et al, 2005; Klemmt et al, 2007). Additionally, increased levels of uPA have been demonstrated in patients with endometriosis. This suggests that both factors may be involved in the degradation of ECM, being a key step in the invasion of endometrial cells and the establishment of lesions (Gilabert-Estellés et al, 2007).

An increase in mRNA and protein levels of PAI-1 in endometriotic tissue have also been observed in comparison with EUT. Increased PAI-1 expression may be involved in the reduction of the invasive potential of endometriotic tissue in advanced stages of the disease (Gilabert-Estellés et al, 2007).

1.5.3. Interplay between ECM remodelling and angiogenesis

From a detailed point of view, the process of angiogenesis can be divided into two stages: the activation phase and the resolution phase. In the activation phase, the extracellular proteolysis is essential for the degradation of the basement membrane, the cell migration and invasion of the ECM. This degradation is mediated by the balance between regulators and effectors. Several components of the ECM are resistant to the action of broad spectrum proteases, but not to the enzymes of the two most relevant families in the degradation of ECM: the plasminogen activator system and matrix metalloproteases (Pepper, 2001; Zorio et al, 2008). Moreover, it has been reported that VEGF-A is capable of inducing uPA expression (Pepper, 2001). Thus, it seems reasonable to simultaneously study components of the angiogenic and fibrinolytic system in our patients.

1.6. Peritoneal fluid

Endometriosis is a multifactorial disease in which endometrial and peritoneal factors such as those related to angiogenesis and proteolysis may be involved (Kobayashi et al, 2000; Braza-Boïls et al, 2013, 2014).

Peritoneum is the tissue that lines the inside of the abdominal cavity, covering the viscera (visceral peritoneum) and the interior of the abdominal wall (parietal peritoneum). In 1922, Novak (Novak, 1922) published an article entitled "About the cause and importance of the physiological ascites in women", in which he first described the presence of a physiological fluid in the peritoneum. It was not until 1980 when Koninckx and co-workers (Knoninckx et al, 1980) proposed peritoneal fluid (PF) as an ovarian exudation product. Actually, PF is defined as "an ovarian exudation product originating mainly as a result of an increased vascular permeability, with cyclic variation in volume and steroid hormones" (Koninckx et al, 1998). This might explain the increase in volume in women with regular menstrual cycle during the follicular phase, the peak in luteal phase and the subsequent declining. Additionally, this also provides an explanation for Koninckx group findings of uniformly low amounts of PF in women with inactive ovaries, women taking combined oral contraceptive pills or postmenopausal women (Knoninckx et al, 1980).

Because ectopic endometriotic lesions located in the pelvic peritoneum are in close relationship with this biofluid, their components have emerged as an important field of study (Koninckx et al, 1998; Cosín et al, 2010; Mier-Cabrera et al, 2011; Na et al, 2011; Liu et al, 2011, Braza-Boïls et al, 2013; Olkowska-Truchanowicz et al, 2013; Rakhila et al, 2016; O et al, 2017; Marí-Alexandre et al, 2017b). PF, which has the function of lubricating the mesothelial wall, is the result of an increased vascular permeability as a consequence of high local concentrations of estrogens and their content is due to both plasma exudate and local secretion (Koninckx et al, 1998). Nevertheless, the content in small molecules (<100 kDa) is similar to that of plasma, due to the process of passive diffusion through the peritoneal surface. On the other hand, larger molecules show a greater difference between their plasmatic and peritoneal concentrations because of the difficulty for them of the diffusion process. Additionally, 17β-estradiol and progesterone steroid hormones have a higher concentration in the PF compared to plasma, because ovarian exudate occurs around the developing follicle and the corpus luteum (Koninckx et al, 1998). This is also the case for patients with benign serous cysts, endometrioma or ovarian malignancies, whose

median concentrations of sHLA-G, IL-10 and TNF-α in PF were higher than serum levels of these markers (Sipak-Szmigiel et al, 2017).

In PF, there exist cell types such as erythrocytes, macrophages and endometrial cells. Endometrial cells secrete products such as glycodelin, the peritoneum substances such as CA-125 and macrophages secrete a large diversity of molecules, among which cytokines, growth factors and angiogenic factors can be found. Due to the local secretion of these substances, their concentration is supra-physiological (Koninckx et al, 1998).

It is well documented that endometriosis is characterized by an important inflammatory process (Bulun, 2009; Augoulea et al, 2012; Reis et al, 2013) with and increased production of ROS (Ngô et al, 2009; Agarwal et al 2012; Polak et al, 2013). Berkes and collaborators (Berkes et al, 2014) and Santulli and collaborators (Santullli et al, 2015) have identified significantly increased levels of protein oxidative stress markers in the PF from women with deep infiltrating endometriosis when compared with endometriosis-free controls. Additionally, NETosis describes the mechanisms by which activated neutrophils expel their entire chromatin, serving as catch and kill scaffold against microorganisms, a structure designated as neutrophil extracellular traps (NETs). Furthermore, it is known that ROS are the major activator of NETosis. The involvement of NETosis in endometriosis was studied by Berkes and co-workers (Berkes et al, 2014), who observed the presence of NET formation in virtually half of the patients with endometriosis, primarily in the stage I and II group and rarely in controls, suggesting that NETosis is implicated in the initiation of the disease. Moreover, the contribution of immune system disorders to endometriosis has been proposed by several authors (Vinatier et al, 1996; Sinaii et al, 2002; Giudice and Kao, 2004; Olovsson et al, 2011). In this context, macrophage migration inhibitory factor (MIF) is arousing growing interest. MIF is a major pro-inflammatory factor found elevated in PF from women with endometriosis. Apart from its effect on activating and inhibiting macrophage mobility, it is also considered a critical upstream activator of innate immunity. MIF may be required for ectopic endometrial tissue growth and progression of endometriosis lesions *in vivo* (Rakhila et al, 2014). Additionally, there is an increased number of peritoneal macrophages, T and B lymphocytes and platelets (Berbic and Fraser, 2011; Olkowska-Truchanowiczet al, 2013; Guo et al, 2016). These cells, among others, are responsible for the elevated levels of cytokines as VEGF-A, IL-6, IL-8, IL-10, IL-17A and TNF-α (Giudice and Kao, 2004; Gilabert-Estellés et al, 2007; Olkowska-Truchanowiczet al, 2013; Vercellini et al, 2014; Rakhila et al, 2016; Jørgesen et al, 2017).

Regarding angiogenesis, VEGF-A has been detected in higher concentrations in the PF of women with endometriosis (McLaren et al, 1996; Gilabert-Estellés et al, 2007; Marí-Alexandre et al, 2017b), being correlated with the disease stage (Shifren et al, 1996). It has been suggested that increased levels of VEGF-A in PF in patients with endometriosis are the consequence of combined expression by ectopic lesions and activated macrophages present in the fluid (McLaren et al, 1996). In addition, some studies indicate that the PF of women with endometriosis induces cell proliferation *in vitro*, although the underlying mechanism is unknown (Minici et al, 2008; Liu et al, 2011).

2. Epigenetics

Initially proposed by AD Riggs and colleagues in 1996, the term epigenetics describes "the study of mitotically and/or meiotically heritable changes in gene function that cannot be explained by changes in DNA sequence". A decade later, A. Bird proposed a refined definition of epigenetics as "the structural adaptation of chromosomal regions so as to register, signal or perpetuate altered activity states" (Bird, 2007). Epigenetic mechanisms are dynamic and reversible, and include: **DNA methylation**, **histone modifications, chromatin remodelling and** expression of **non-coding RNAs (ncRNAs)** (Portela and Esteller, 2010).

Nowadays, a growing body of evidence suggests that epigenetics could be involved in the pathophysiology of endometriosis (Guo, 2009), with an exponential increase of papers published on this issue in recent years.

2.1. miRNAs

miRNAs are small (19-22 nt) non-coding RNAs that can act as post-transcriptional regulators of gene expression, reducing the expression of their target mRNAs either by inhibiting its translation or by promoting its degradation. miRNAs usually regulate gene expression by binding to the 3' UTR (UnTranslatedRegion) of their target mRNA. Importantly, several miRNAs can target a given mRNA and a single miRNA can target several mRNAs, increasing the complexity of the regulatory mechanism mediated by these molecules (Lee et al, 1993; Lee et al, 2003; Bartel, 2004; Teague et al, 2010; Caporali et al, 2011). In malignancies, miRNAs can act as oncogenes or tumour suppressors, depending on their targets (Gailhouste et al, 2013; Nohata et al, 2013; Huang et al, 2014). It is important to highlight that the miRNA expression profiles are specific of tissue and cell type (Bartel, 2004). To date, more than 1881 miRNA precursors, coding for more than 2500 mature miRNAs have been described in humans (Kozomara et al, 2014). miRNAs were first described in 1993 by Lee and collaborators (Lee et al, 1993) in the worm *Caenorhabditis elegans*. Since then, studies about biogenesis, functions, roles and characterization of the mechanism of action of miRNAs have grown considerably and nowadays they are considered as pathophysiological players and/or excellent biomarkers of a myriad of diseases such as coronary artery disease (Zorio et al, 2009; Papageorgiou et al, 2013; Economou et al, 2015; Braza-Boïls et al, 2016; Marí-Alexandre et al, 2017c), cancer (Schwarzenbach et al, 2014; Cheng et al, 2015), and several gynaecological pathologies, including endometriosis (Fassbender et al, 2013, 2015).

2.1.1. Canonical pathways of miRNAs biogenesis

In the canonical pathway of miRNA biogenesis, the pri-miRNAs transcribed by RNApol II undergo several sequential cleavage processes, both in the nucleus and in the cytoplasm, to finally give rise to mature miRNAs (**Fig. 7**). The first of these cleavage processes occurs in the nucleus, thanks to the action of the RNAse III-enzyme "Drosha", with the help of a double stranded RNA binding protein: DGCR8.

This protein complex is known as the *microprocessor*, and produces stem-loop precursor of about 70 nucleotides, called pre-miRNA, in which two nucleotides protrude from the structure at the 3' end, while there exist a protruding phosphate group at the 5' end. At this

point, the pre-miRNA is translocated to the cytoplasm thanks to the action of Exportin-5, a Ran-GTP dependent protein (Lund et al, 2004; Meijer et al, 2014). From this step on, additional processing is common for miRNAs and exogenous siRNAs.

Once in the cytoplasm, the pre-miRNA is cleaved by another RNAse III-enzyme, called "Dicer", in combination with the TRBP (Machlin et al, 2011). As a consequence, the loop sequence of the hairpin is released and Dicer produces a duplex RNA of about 22 nucleotides with small protruding 3' ends. This step is of vital importance as it defines the 3' end of the 5' strand and the 5' end of the 3' strand (Eulalio et al, 2008). Then, "Dicer" transfers RNA-duplex to an Ago (Argonaute) protein, which forms the nucleus of the RNA-induced silencing complex (RISC), called the pre-RISC complex at this point.

The mature RISC complex is achieved once one of the two duplex strands is removed, a process called "strand selection". The main determinant of this process seems to be a thermodynamic factor, mainly involving the first four nucleotides of the duplex. Therefore, the weaker interaction end will preferably be unrolled and remain as the "guide strand", while the so-called "passenger strand" will be discarded (Meijer et al, 2014). Mature RISC complex is able to "scan" the cytoplasm searching for mRNAs capable of pairing with the loaded miRNA.

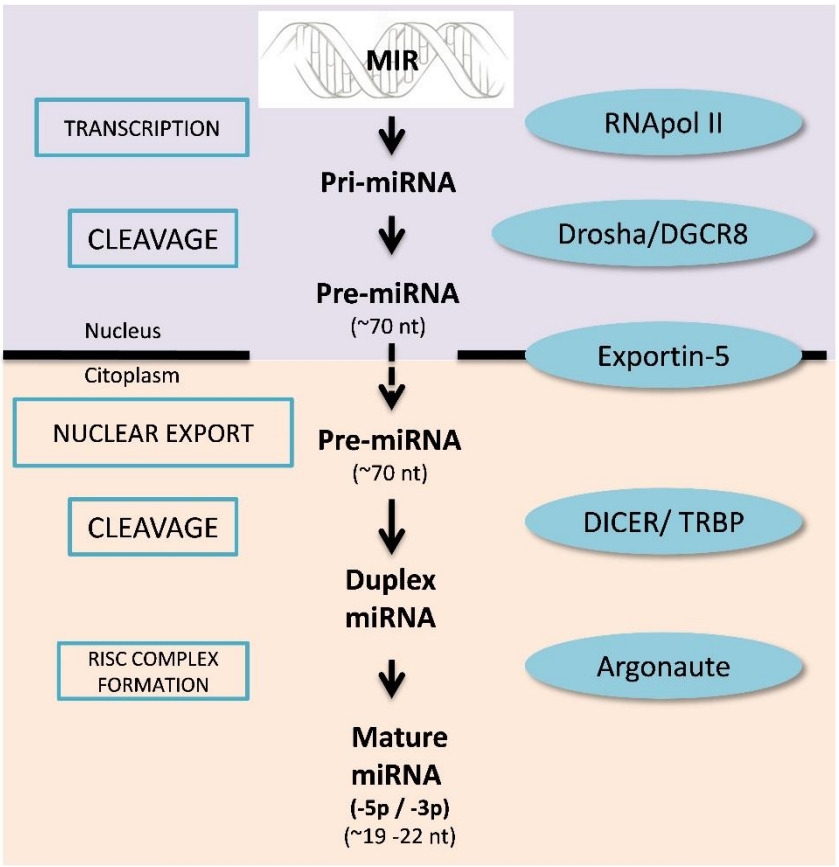


Figure 7. Schematic representation of the canonical pathway of biogenesis of miRNAs. DGCR8: Di George Syndrome Critical Region 8; RISC: RNA-induced silencing complex. TRBP: trans-activation response RNA-binding protein.

2.1.2. Non-canonical pathways of miRNAs biogenesis: the "mirtron pathway"

Apart from the canonical pathway of miRNA biogenesis, intronic miRNAs can undergo the "mirtron pathway". In this non-canonical pathway of biogenesis, once the splicing process occurs, the intron has a looped form, in which the 3' branch point is linked to the 5' end of the intron. The action of the enzyme Ldbr (lariat debranching enzyme) allows this loop to become a pre-miRNA, which can enter into the canonical biogenesis pathway of the miRNAs (Westholm and Lai, 2011; Desvignes et al, 2015). Since the vast majority of intron miRNAs are in the sense of the strand, it seems plausible to think that their expression may be related to that of the host mRNA (Rodriguez et al, 2004; Baskerville and Bartel, 2005) in terms of specificity of tissues and relative abundance (Gregory and Shiekhattar, 2005).

With the avenue of new sequencing technologies, the field of miRNA research has undergone an unprecedented expansion in terms of discovery of genetic origins, biosynthetic pathways and sequence variants. As a result, several ncRNAs have been identified as additional sources of miRNAs, including snoRNA, lncRNA, and tRNA genes with both dependent and independent biogenesis of Drosha and/or Dicer (Desvignes et al, 2015).

2.1.3. miRNA nomenclature

The recent advances in high-throughput sequences applied to the miRNA discovery have enormously challenged criteria for miRNA annotation. Nomenclature rules are currently defined by miRbase v.21 (Kozamara et al, 2014) and the mature form of the miRNA fit the form hsa-miR-XX-3p/-5p, where the prefix refers to the species (e.g., hsa- for *Homo sapiens*). When it is written in capitalized letters, “MIR”, it refers to the gene; and pre-miRNA and pri-miRNA are named as “mir-”. Distinct precursor sequences and genomic loci expressing identical mature sequences get names of the form hsa-mir-121-1 and hsa-mir-121-2 and adding letters as suffixes denotes mature sequences closely related (hsa-miR-121a and hsa-miR-121b) named miR families. Cloning studies sometimes identify two mature sequences originated from the same pre-miRNA. The ratio between the two opposite mature strands can vary depending on developmental stage, being differentially expressed in distinct tissues or cell types, as well as in pathological conditions (Meijer et al, 2014; Desvignes et al, 2015). Previous nomenclature versions named less expressed strand with an asterisk (*) (hsa-miR-XX*). However, since recent studies have demonstrated that both strands are functional and the ratio between them depends on the cellular type or status, the annotation criteria has been appropriately changed to the current -3p/-5p end. Apart from the aforementioned miRNA nomenclature, miRBase also identifies mature miRNAs with a MIMAT accession number. From our own experience, we do recommend authors to refer studied miRNAs in their manuscripts with the current -3p or -5p suffix and also to include the miRBase MIMAT reference and oligonucleotide sequence in order to avoid future misunderstanding that further nomenclature modifications could introduce.

In the light of current discoveries in miRNA origins, biosynthetic pathways, and sequence variants, Desvignes and co-workers proposed a revised miRNA nomenclature criterion in the aim of encompassing recent findings in the field. The authors proposed to modify

nomenclature not only based on biogenesis but also on their function (Desvignes et al, 2015).

2.1.4. Determination of the miRNA:mRNA pairing

The miRNA: mRNA pairing is defined by Watson-Crick interactions between the 3' untranslated region (3'-UTR) of the mRNA and a short region of nucleotides spanning positions from 2 to 8 of the miRNA, known as the "seed sequence" (further discussed in section 2.1.5.). It is important to mention that a single miRNA can bind to several target mRNAs, and in turn, that several miRNAs can bind at different positions of the 3'-UTR end of the mRNA, increasing the complexity of the regulatory mechanisms that mediated by miRNAs (**Fig. 8**).

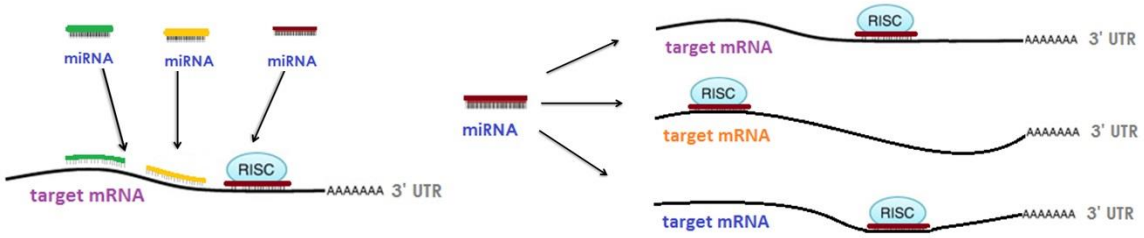


Figure 8. Schematic representation of the mechanism of action of mature miRNAs. UTR: untranslated region

However, beyond this general principle of miRNA:mRNA interaction, miRNA:mRNA paring with the 5'-UTR have also been described, such linkages being of clinical interest, for example, in infection by the Hepatitis virus C (Machlin et al, 2011), and that other nucleotides outside the seed sequence may also contribute to the determination of the mRNA fate (Eulalio et al, 2008).

Once the miRNA:mRNA binding is achieved, the silencing of the mRNAs can be achieved by cleavage or by translational repression thereof. This is conditioned by the degree of complementarity between the miRNA and its target mRNA: if this is perfect, what occurs is the cut of the mRNA mediated by Ago, approximately in the centre of the miRNA length; while imperfect pairing mediates translational repression of mRNA by different mechanisms, such as inhibition of translation elongation, co-translational protein degradation, competition for cap structure, inhibition of ribosomal subunit binding , the inhibition of circularization of mRNA by deadenylation and deadenylation and decapping (Eulalio et al, 2008).

2.1.5. Importance of the "Seed sequence"

miRNAs exert their function by complementary Watson-Crick binding between the miRNA and the 3'-UTR end of the target mRNA. However, despite its small size, not all the length of the miRNA is involved in this interaction. Instead, there is a region of about 7 nucleotides (from 2-8 in the 5' →3' sense) called "seed region" (**Fig. 9**), which is the most important for mating and to define the target mRNAs (Ambros, 2004; Grimson et al, 2007).

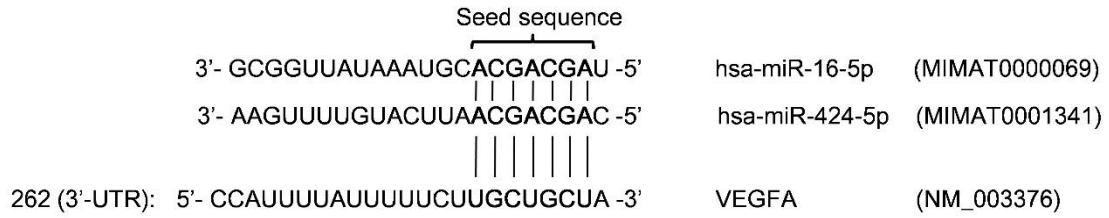


Figure 9. Illustrative example of miRNA seed sequence:mRNA binding. miR-16-5p and miR-424-5p share the same seed sequence (nt 2 to 8, 5' → 3' sense) and also their target genes (e.g. *VEGFA* mRNA). UTR: Untranslated region; VEGFA: Vascular endothelial growth factor A.

Dr. Bartel provided an explanation for the importance of nucleotides in position 2 to 8, based on the geometry of the single stranded miRNA inside the RISC (Bartel, 2009). Thus, whereas nucleotide in position 1 is twisted away from the helix and permanently unavailable for pairing, nucleotides 2–8 are preorganized to favour efficient miRNA:mRNA pairing. Finally, nucleotides 9–11 are facing away from an incoming mRNA and unavailable for nucleation, and the remainder of the miRNA is bound in a configuration that is not preorganized for efficient pairing.

Remarkably, miRNAs encoded in different chromosomes, belong to the same family, because they share the same "seed region" (Kamanu et al, 2013). They have probably reached different chromosomes by gene duplication during evolution, establishing them as miRNAs analogues (e.g., miR-17 and miR-18a).

Recently discovered, it is important to note that the miRNA sequence does not always correspond to the complementary sequence codified in the DNA. Instead, up to 6% of the miRNAs have been described to undergo an "editing" process. This implies that a single pre-miRNA can render multiple mature miRNAs, which differ in their length and sequence, giving rise to the so-called **isomiRs**. Since the seed sequence is by definition the nucleotide sequence between positions 2 to 8, the editing process may alter the "seed sequence", conferring the miRNA affinity for other target mRNAs, (Desvignes et al, 2015; Neilsen et al, 2012).

2.1.6. Databases for miRNA research

Freely available miRNA databases for miRNA annotation and target genes search are essential tools in the field of miRNA research.

- **miRBase** (http://www.mirbase.org/) (previously called "the microRNA Registry") is the public repository for miRNA sequences since 2002. This database is managed by the laboratory of Dr. Griffith-Jones, at the University of Manchester (UK), and is continuously updated (release 21 launched at July 2014 containing 28645 entries representing hairpin precursor miRNAs, expressing 35828 mature miRNA products, in 223 species). For a miRNA sequence to entry, an article describing their identification must have been accepted in a peer-review journal. The importance of miRBase is such that newly developed technologies rely on annotations in this database to build their specific probes.

- **Databased for miRNA:mRNA interaction prediction:**12 databases have been generated for miRNA:mRNA interaction prediction, such as **microRNA.org** (http://www.microrna.org), **TargetScan** (http://www.targetscan.org/vert_71/) or **miRDB** (http://mirdb.org), to mention a few. Although all of them have a high degree of overlap, differences are the consequence of alignment artifacts, the use of different UTR or miRNA sequences, besides differences to the prediction algorithm themselves.

- **Databases for validated mRNA targets:** 4 manually curated databases incorporate information of experimentally validated miRNA:mRNA interactions:

    - ✓ **miRTarBase** (http://mirtarbase.mbc.nctu.edu.tw/).
    - ✓ **PhenomiR** (http://mips.helmholtz-muenchen.de/phenomir/)
    - ✓ **miR2Disease** (http://www.mir2disease.org/)
    - ✓ **HMDD** (http://www.cuilab.cn/hmdd).

In recent years, a new resource called **miRWalk** (http://mirwalk.uni-hd.de/) has provided an integrated view of all information generated from the aforementioned databases.

Anyway, what is clear from the avalanche of data exponentially generated in recent times is that bioinformatics is called to play an indispensable role in the field of biology in general and in the field of miRNAs in particular, to shed light on biologic pathways regulated by differently expressed miRNAs in different pathological conditions.

2.1.7. miRNAs in endometriosis

A systematic PubMed search restricted to papers published in English with the key words "miRNA and endometriosis" yielded a result of 120 published papers (**Fig.10**) (in PubMed, recovered on October, 6th 2017 at https://www.ncbi.nlm.nih.gov/pubmed/).

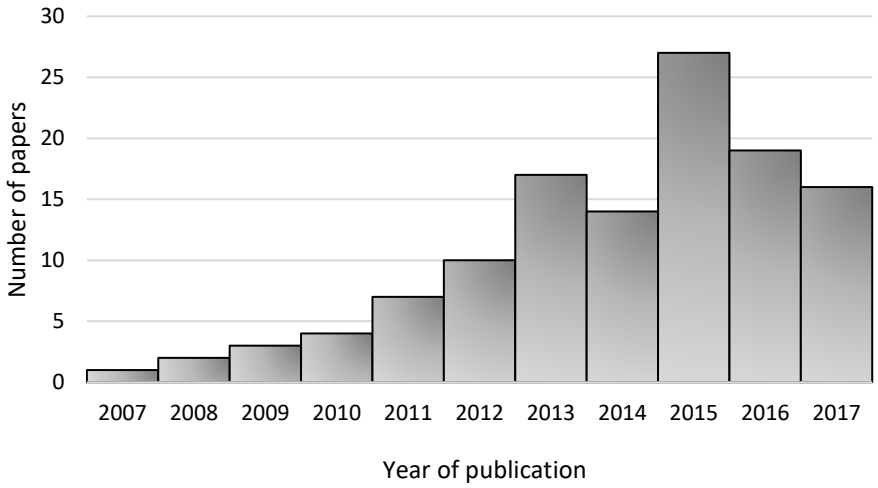


Figure 10. Histogram showing the frequency per year of published papers on miRNAs and endometriosis since the first publication a decade ago.

Recently reviewed (Marí-Alexandre et al, 2016; Borghese et al, 2017; Saare et al, 2017), 10 studies have employed different high-throughput methodologies to evaluate distinct miRNA profiles in endometriosis (Burney et al, 2009; Ohlsson-Teague et al, 2009; Filigheddu et al, 2010; Hawkins et al, 2011; Laudansky et al, 2013, 2015; Braza-Boïls et al, 2014; Saare et al, 2014; Shi et al, 2014; Yang et al, 2016). These studies have evaluated differences between EUT and CNT endometrium (Burney et al, 2009; Laudanski et al, 2013, 2015; Braza-Boïls et al, 2014), OMA vs EUT (Filigheddu et al, 2010; Hawkinset al, 2011) and also CNT endometrium (Braza-Boïls et al, 2014), and PIs vs EUT endometrium (Ohlsson-Teague et al, 2009; Saare et al, 2014). Nevertheless, two studies (Shi et al, 2014; Yang et al, 2016) do not specify the type of endometriotic lesions analysed. This lack of information prevents extracting conclusions from them, given the special features of each type of endometriotic lesion (See section 1.2.)

Additionally, other studies have been focused on candidate miRNAs analysis to assess miRNA expression in endometrial and endometriotic tissues (Ramon et al, 2011; Shen et al, 2013; Zheng et al, 2014; Dong et al, 2015; Graham et al, 2015; Long et al, 2015), and many others employ *in vitro* (Petracco R et al, 2011; Dai et al, 2012; Adammek et al, 2013; Braza-Boïls et al, 2013; Xu et al, 2016; Park et al, 2017) and animal models (Nothnick et al, 2014; Hsiao et al, 2015) to assess differential miRNA expression in endometriosis.

In recent years, the evaluation of circulating miRNAs as biomarkers is attracting great interest (reviewed in Marí-Alexandre et al, 2016), although this far from the scope of the present work.

All the aforementioned papers propose a regulatory function for miRNAs in a myriad of processed involved in the pathophysiology of endometriosis, such as angiogenesis, proteolysis, extracellular matrix remodelling, inflammation, proliferation or apoptosis. Nevertheless, the lack of overlap among different studies is noticeable. Prof. Dr. Borghese and co-workers proposed several reasons for this, as differences in study cohorts, disease severity and classification, the different menstrual cycle phases and circadian rhythm of miRNA expression (Borghese et al, 2017). Additionally, the housekeeping gene chosen as normalizer and the different technologies employed for both discovery and validation phases could help understanding differences among published papers (Saare et al, 2017).

2.2. DNA methylation

Among epigenetic marks, DNA methylation is by far the most widely studied. As a basic concept, this covalent modification takes place at the 5' carbon of cytosines, a process catalysed by a family of enzymes: the DNA-methyltransferases (DNMTs). Whereas *DNMT1* recognizes hemimethylated DNA and is classified as maintenance methyltransferase, *DNMT3a* and *DNMT3b* can introduce methylation marks without a template and are therefore termed *de novo* methyltransferases. Deviations in DNA methylation patterns can occur via gain (hypermethylation) or loss (hypomethylation) of methylation marks with respect to methylomes defined as normal (Portela and Esteller, 2010). In a cancer context, hypermethylation at CpG islands of gene promoters is associated with the repression of tumour suppressor genes (TSGs) expression (Greger et al, 1989; Sandoval et al, 2012). Conversely, genome-wide hypomethylation in cancer cells has been linked to expression of proto-oncogenes, genomic instability (Sheaffer et al, 2016) and

malignant transformation of tumours, a feature that increases with cancer progression (Esteller, 2008).

2.2.1. DNA methylation in endometriosis

In contrast with the vast available literature in cancer research, the role of DNA methylation alterations in the pathophysiology of endometriosis has just begun to be explored.

2.2.1.1. DNA methylation across the menstrual cycle in the healthy endometrium

In a seminal paper published in 2014, Houshdaran and co-workers (Houshdaran et al, 2014) determined that the DNA methylome of endometrium is variable across the menstrual phase and is associated with gene expression regulation. They performed whole-genome DNA methylome interrogation with the Illumina Infinium Human-Methylation27 DNA methylation BeadChip assay (See section 3.2.2. for further details) in 18 endometria samples (n=6 in proliferative, n=6 in early secretory and n=6 in midsecretory phases; menstrual phase excluded) from non-endometriosis women. The rationale behind this study was to assess if DNA methylation could be involved in the regulation of gene expression changes across the menstrual cycle, in addition to the known regulation by oestradiol- and progesterone-dependent transcription factors. As a result, authors observed segregation of proliferative from secretory phase samples by unsupervised cluster analysis of differentially methylated genes. Comparison of changes in transcriptomes and corresponding DNA methylomes from the same samples revealed association of DNA methylation and gene expression in loci important in endometrial biology. Additionally, some of these changes were validated in an *in vitro* model.

Later on, Kukushkina and collaborators (Kukushkina et al, 2017) employed the Infinium HumanMethylation450K BeadChip (See section 3.2.2. for further details) to analyse DNA methylation changes associated with transition to pre-receptive (early-secretory phase, day 2 after ovulation) to receptive phase (midsecretory phase, day 8 after ovulation). To the best of my knowledge, this is the first study in which endometria at two time-points from the same women within a menstrual cycle have been analysed. Although authors observed small-scale changes affecting 5% of the studied CpGs, seven differently methylated genes (namely KRTAP17-1, CASP8, RANBP3L, WT1, MPP7, PTPRN2, and HCP5) had also been reported in previous studies (Houshdaran et al, 2014; Saare et al, 2016).

2.2.1.2. DNA methylation in eutopic versus control endometrium

Apart from papers evaluating DNA methylomes in primary stromal cell cultures from EUT and CNT endometria (Yamagata et al, 2014; Yotova et al, 2017), only three papers analysing the changes in DNA methylation patterns in the endometrial tissues from women with and without endometriosis have been published (Naqvi et al, 2014; Saare et al, 2016; Houshdaran et al, 2016). However, since this issue represents a specific Chapter of the present work (see Chapter 4), a detailed description and discussion of these results will be given thereafter.

2.2.1.3. DNA methylation in ectopic versus eutopic endometrium.

Dr. Yamagata and collaborators also considered including in his previously mentioned study primary stromal cell cultures from OMA (Yamagata et al, 2014). Clustering analysis segregated these cells from those of both endometrium, with 515 hypomethylated CpGs (corresponding to 441 genes) and 368 hypermethylated CpGs (corresponding to 329 genes), related to signal transduction, development and cell adhesion processes ($|\Delta\beta|>0.2$).

Although with different experimental designs, up to now only two studies (apart from the aforementioned Dr. Yamagata's oral communication) have analysed whole-genome methylome in ectopic *vs.* endometrial tissues (peritoneal implant, deep infiltrating endometriosis and OMA *vs.* EUT endometria in Borghese et al, 2010, focused on promoters; and OMA *vs.* CNT in Dyson et al 2014), by means of either meDIP-on-chip or the Illumina Infinium HumanMethylation450k BeadChip assay, respectively. Whereas the former study identified hypermethylated regions mainly located at the ends of the chromosomes, the latter identified 42,248 differentially methylated CpGs in endometriosis compared to healthy cells, corresponding to 403 genes, mainly of transcription factors, highlighting HOX gene clusters, nuclear receptor genes, and the GATA family of transcription factors.

3. Techniques for epigenetic and epigenomic analyses

In the field of biomedical research, the most common goal in miRNA studies is to determine what miRNAs are deregulated in biological specimens from a patient cohort, in comparison to specimens from a cohort considered control. Initially, a *screening or discovery phase* is performed by means of high-throughput technologies (see Section 3.1.1.); this allows to interrogate the expression of thousands of miRNAs, albeit due to the high economic cost, only a few samples are usually analysed in this step. Secondly, a *validation phase* is required to corroborate the previously obtained results in an extended cohort of samples. In this step, the most commonly used technique is qRT-PCR, as will be discussed hereafter (see Section 3.2.1) (Marí-Alexandre et al, 2016).

3.1. Techniques for epigenomic analyses

3.1.1. Techniques for epigenomic analyses of miRNAs

Nowadays, two techniques account for those assessing miRNA expression profiles: *miRNA microarrays* and *Next Generation Sequencing* (NGS).

- *miRNA microarray platforms:*

Microarrays platforms have meant a revolution in the field because of its ability to simultaneously detect thousands of molecules in a single experiment. This technology is based on the link of complementary probes on glass, quartz or nylon supports, followed by the hybridization of fluorescently labelled samples. After a normalization step to eliminate the background noise of the reaction and differences in the hybridization process, the relative signal on each probe reflects the original amount of miRNA in the studied sample (Usó et al, 2014).

Since hundreds of new ncRNAs are described on an annual basis, miRNA microarray platforms (from companies such as Affymetrix, Agilent, Illumina and Nanostring) must be continuously updated (Mestdagh et al, 2014; Marí-Alexandre et al, 2016).

- *Next Generation Sequencing:*

NGS technology has permitted to overcome the limitations in throughput, scalability, speed and resolution of capillary electrophoresis-based Sanger sequencing (also known as first-generation sequencing). The ability to read millions of short fragments of DNA was soon moved into a new methodology, RNA-seq, which includes RNA sequencing and quantification. Total RNA from samples is digested and retrotranscribed through ligation of universal primers. cDNA products are then ligated to primer adaptors for NGS and amplified and isolated in single sequencing reactions on a surface containing millions of "nanopore" sequencing reactors. Second generation sequencers (from companies such as Roche Diagnostics, Life Technologies and Illumina) are able to detect millions of isolated sequencing reactions and to generate data related to nucleotide sequences and the number of reads of each sequence. Importantly, NGS permits to distinguish between miRNAs that differ by a single nucleotide and isomiRs (variants of the same miRNA). The bioinformatic software associated with the NGS sequencer is able to align each short RNA fragment with their corresponding genomic region, building a complete transcript read. The number of

reads obtained correlates to the original amount of RNA in the sample, together with sequence variant detected in the alignment (Marí-Alexandre et al, 2016).

Nevertheless, data generated by these technologies requires a validation phase, being qRT-PCR the technique of election (see Section 3.2.1) (Mestdagh et al, 2014).

3.1.2. Techniques for epigenomic analyses of DNA methylation

Nowadays, the use of the bisulphite treatment of the DNA implemented with hybridization in a matrix platform has displaced the use of strategies based on the enrichment of methylcytosine to study global epigenetic patterns such as 1) the methylated CpG islands recovery assay (***MIRA***) (Jiang et al, 2004), 2) the immunoprecipitation assay of methylated DNA and microarray (***MeDIP-on-chip***) (Jacinto et al, 2007) and 3) HpaII methylation-sensitive restriction enzyme (***HELP***) coupled with PCR-mediated ligation PCR (***LM-PCR***) (Suzuki and Greally, 2010).

The first developed matrix for analysing methylation profiles in bisulphite converted samples (see Section 3.2.2. and **Fig. 11**) was the ***Illumina Golden Gate® assay***, which interrogated the methylation status of up to 1,536 targeted CpG sites of 371 genes related to cancer. Later on, the ***Illumina Infinium Human Methylation27K BeadChip assay*** reached the market, which was able to analyse the methylation status of 27,578 CpG sites, comprising of 14,475 genes (67%) RefSeq Genes, including 110 miRNA promoters. A great advance in the analysis of DNA methylation status was achieved with the arrival of the ***Illumina Infinium Human methylation450K BeadChip assay***. This platform interrogates at a single base resolution more than 450,000 CpGs (specifically, 485,764 CpGs), covering 99% of the genes annotated in RefSeq (21,233 genes in total, including ncRNAs), with an average of 17 CpG sites per gene region distributed through the promoter, 5' UTR end, the first exon, the gene body, and the 3'UTR region; and also 96% of the CpG islands (CGI) and CpG shores in the genome (Bibikova et al, 2011; Sandoval et al, 2011). Regarding the distribution of functional genomics, the probes are located mainly in promoters and intergenic regions, although it is also important to highlight the presence of miRNA promoter regions. Microarrays have been shown to be useful in addressing the study of DNA methylation profiles at the genomic level in a large cohort of patients, due to their reproducibility, rapidity and reasonable price per sample. However, as a principal disadvantage we could cite this assay (450K) does not cover the whole genome (Bibikova et al, 2011; Sandoval et al, 2011).

At the time we planned to perform our analysis, a new version of the platform replaced the 450k assay: the Infinium MethylationEPIC BeadChip from Illumina. This system covers more than 850,000 CpG methylation sites (850K) with more than 90% of the 450K sites plus an additional 333,265 CpGs located in enhancer regions identified by the ENCODE and FANTOM5 projects. Additionally, this latter platform has a high reproducibility in the analysis of fresh frozen and formalin-fixed and paraffin-embedded samples (Moran et al, 2016).

It is also currently possible to perform a rapid unbiased analysis of the total DNA methylome at a single base resolution by means of whole genome sequencing (Ziller et al, 2015). Although the use of NGS in methylome studies has increased in the last 2 years,

most translational cancer studies are still being performed using microarray platforms, primarily due to the excellent cost/quality ratio.

All these technological improvements have so far permitted to overcome previous limitations, suggesting that in the short-term researchers will be able to reveal the state of methylation in different pathologies of the 28 million of CpG dinucleotides present in the human methylome.

3.2. Techniques for epigenetic analyses

3.2.1. Techniques for epigenetic analyses of miRNAs

- *Quantitative Real-Time Polymerase Chain Reaction (qRT-PCR)*

qRT-PCR is a fast, easy, and affordable technique for quantification of miRNAs, which allows working with very few amounts of starting RNA template. The first step in a qRT-PCR experiment consists in the synthesis of DNA complementary to miRNA through an adapter following a retrotranscription protocol. The subsequent exponential amplification process in qRT-PCR is an extremely sensitive and accurate method for detecting molecules at very low level. Pre-amplification and amplification increase the sensitivity of quantification, but can be biased in low expressed sequences due to diverse protocol steps. Nevertheless, normalization can be challenging because "housekeeping" molecules commonly used as normalizers might be not stably expressed in some models or pathologies (Marí-Alexandre et al, 2016).

In any case, so far, qPCR is the reference method for expression validation of other techniques in miRNA research (Zampetaki and Mayr, 2012; Mestdagh et al, 2014).

Nowadays, some high-throughput PCR designed plates, called arrays, have been developed by some companies such as Applied, Qiagen and Exiqon. They include a customized selection of ready-to-use probes in order to quantify a high number of miRNAs from a single sample reducing technical variations (Marí-Alexandre et al, 2016).

- *In situ hybridization (ISH)*

Although not employed in this work, it is worthy to mention that some companies (Exiqon, Merk-Millipore) have also developed fluorescent or antibody-conjugated coloured-coupled probes for *in situ* miRNA detection. These methodologies allow localizing miRNA molecules in cells or tissues, helping to better characterize its biogenesis, pathways, and activity (Marí-Alexandre et al, 2016). Fluorescent-labelled probes show miRNA localization in fixed or live cells and allow, in some assays, to perform flow sorting of cells expressing specific regulators (Urbanek et al, 2015).

3.2.2. Techniques for epigenetic analyses of DNA methylation

During the late 1980s and 1990s, two different strategies for evaluating DNA methylation patterns in organisms were developed: restriction enzyme-based methods (Bird and Southern, 1978) and approach of candidate genes after bisulphite conversion (Wang et al, 1980):

- On the one hand, restriction enzyme-based methods used different techniques to analyse DNA methylation in a wide-ranging genome: *Restriction Site Genomic Scanning* (***RGLS***) method (Hatada et al, 1991) and *amplification of intermethylated sites* (***AIMS***) (Firgola et al, 2002). All of these tools took advantage of methylation-sensitive restriction enzymes to analyse a limited number of genomic sites.

- On the other hand, the advent of bisulphite conversion was meant a milestone in epigenetic research. Through this chemical reaction, unmethylated cytosine residues are transformed into uracil, but do not affect 5-methylcytosine (**Fig. 11**). The implementation of this technique with genomic sequencing or PCR amplification (*methylation specific PCR*, ***MSP***) allowed a rapid and sensitive interrogation for DNA methylation at any target sequence suitable for the identification of DNA methylation alterations in selected candidate genes. Furthermore, bisulphite genomic sequencing is the method of choice for validation of epigenomic data.

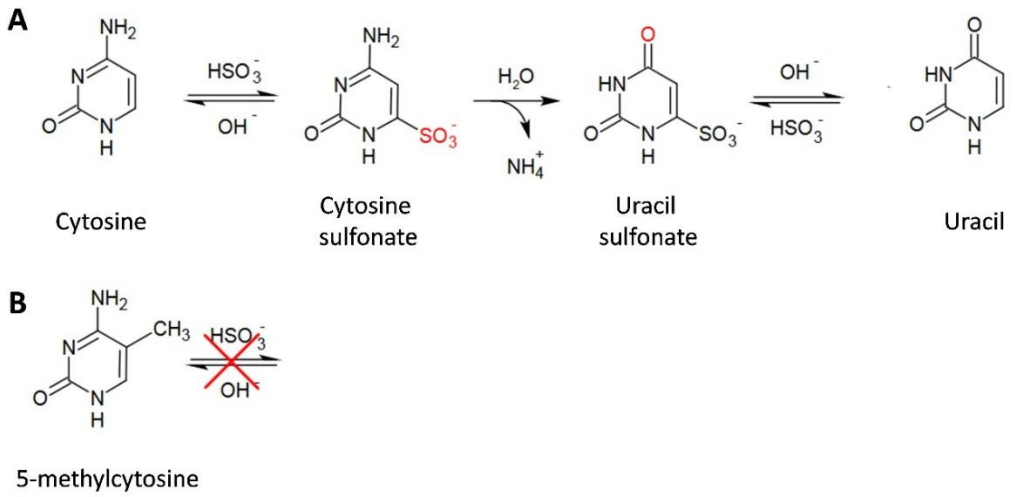


Figure 11. Chemical modifications in the bisulphite conversion reaction. This technique allows the conversion of A) unmethylated cytosine into uracil, while B) cytosines methylated in the 5' position remain insensitive to this chemical modification.

4. Epigenetic editing

With the aim of reversing the epigenetic alterations that trigger disease-specific events, several epigenetic therapies have been developed based on both untargeted and targeted approaches. In untargeted strategies, the catalytic activity of the enzyme responsible for the epigenetic modification is blocked, and consequently the effect spreads throughout the genome. Currently, this group is primary represented by DNA methylation inhibitors and histone deacetylase inhibitors (Vendetti and Rudin, 2013; Mottamal et al, 2015). In contrast, in targeted approaches direct epigenetic modifications are made to specific loci in the genome with a local effect. For this, fusion proteins consisting of a DNA binding domain (DBD) providing locus specificity and an effector domain facilitating the chemical modification are required (Jurkowski et al, 2015). As will be described below, locus specificity is currently achieved with zinc finger proteins (ZFP), transcription activation-like effector (TALEs) proteins and CRISPR-Cas9 (clustered, regularly-interspaced short palindromic repeats (CRISPR)-associated protein 9) (Jurkowski et al, 2015). Furthermore, the effector domain can be represented either by different enzymes (DNMT, HMT, HDAC, etc) (Falahi et al, 2013), or by transcription activators or repressors, suchas the viral protein VP64 or super KRAB domain (SKD) (Huisman et al, 2015; Garcia-Bloj et al, 2016). Additionally, miRNA mimics and antimiRs represent an active field of research in targeted epigenetic editing scenario.

4.1. Untargeted therapies: DNA methylation inhibitors

Initially developed as a cancer treatment, several strategies targeting the methylation machinery have been developed with the aim of restoring TSG gene expression and preventing tumour proliferation (Oronsky et al, 2014), provided the common finding of TSG promoter hypermethylation in cancer cells and tumour biopsies. Nowadays, this therapeutic approach is represented by the hypomethylating agents (HMAs) 5-azacytidine and 2'-deoxy-5-azacytidine. Once incorporated into nucleic acids, these chemical analogues of cytidine covalently and irreversibly bind to DNMTs, causing global DNA hypomethylation and DNA damage induction (Christman, 2002; Hollenbach et al, 2010) (**Fig. 12**).

The first successful clinical application of an untargeted epigenetic therapy was achieved in haematological tumours. As a consequence, both 5-azacytidine and 2'-deoxy-5-azacytidine were approved by the Food and Drug Agency of the USA and the European Medicines Agency as Vidaza™ and Decitabine™ or Dacogen™, respectively, for the treatment of Acute Myelogenous Leukaemia, the most prevalent acute leukaemia in adults characterized by an epigenetic dysfunction (Gallipoli et al, 2015). Importantly, these compounds are currently the first therapeutic choice for patients with myelodysplastic syndromes who are not candidates for allogenic hematopoietic stem cell transplantation (Finelli et al, 2016), and several clinical studies are evaluating their curative possibilities in solid malignancies (NCT01193517; NCT02316028; NCT02795923; NCT00978250).

With regards to endometriosis, these compounds have only been used as an experimental tool for *in vitro* data validation (5-azacytidine: Lu et al, 2013; 2'-deoxy-5-azacytidine: Izawa et al, 2008; Xue et al, 2007a, 2007b; Yotova et al, 2017).

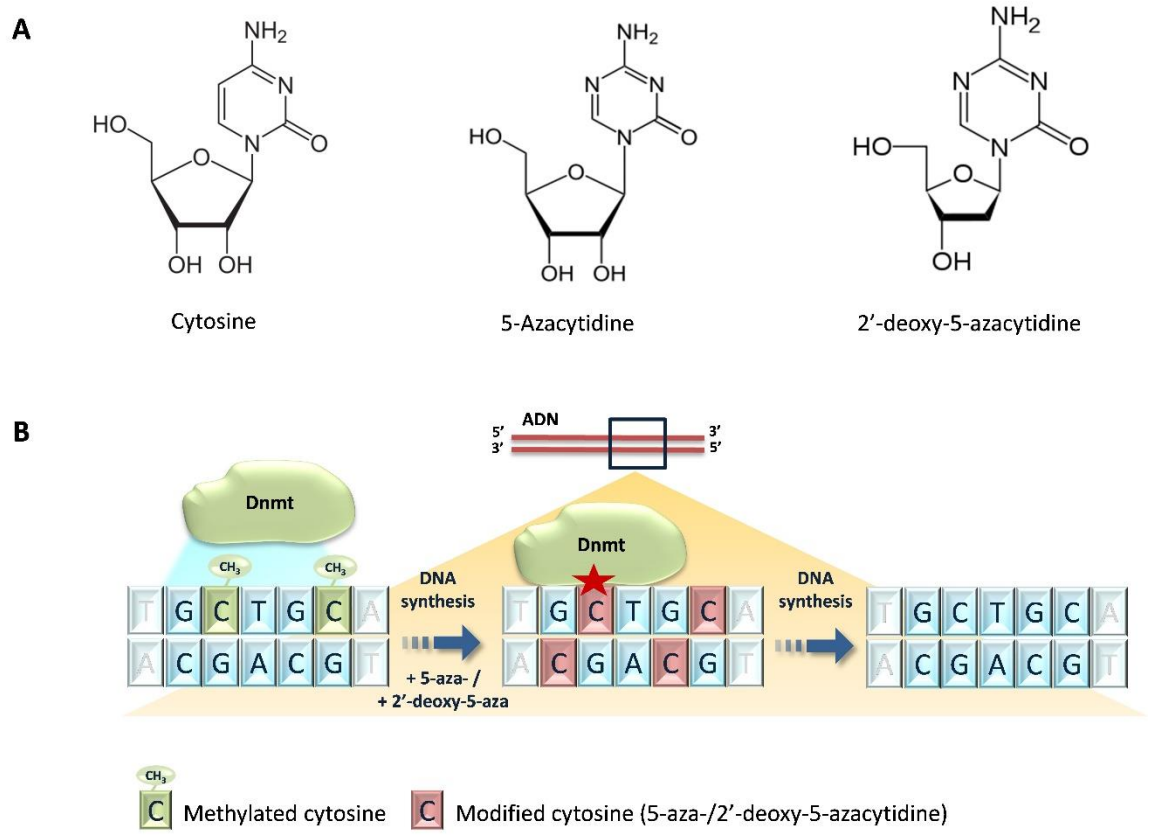


Figure 12. Mechanism of action of hypomethylating agents. A) Chemical structure of the main hypomethylating agents, 5-azacytidine and 2'-deoxy-5-azacytidine, compared to the cytosine nitrogenous base. B) Methylation at cytosines in DNA is achieved by the action of Dnmt enzymes (e.g. *Dnmt3a*). This renders a compacted chromatin structure and a repressed transcriptional state. Through several cell divisions, cytosine analogues such as 5-azacytidine and/or 2'-deoxy-5-azacytidine are incorporated across the genome, thus covalently and irreversibly binding to Dnmts. As a consequence, active Dnmts enzymes are depleted in cells, and hypomethylation takes place throughout the genome. Dnmt: DNA methyltransferase, CH_3: methyl group; 5-aza: 5-azacytidine; 2'-deoxy-5-aza: 2'-deoxy-5-azacytidine (**Annex 1, Paper 4**).

4.2. Targeted therapies: Zn finger proteins and CRISPR/dCas9

Targeted epigenetic editing field is an incipient area of research, less developed than genetic editing from which epigenetic tools derive. To our knowledge, no single publication has reported specific achievements in endometriosis to date. However, some successful studies that anticipate the clinical application of targeted epigenetic editing are worthy of mention. With regard to Transcription Activator-like Effectors (TALEs), several inherent limitations (such as susceptibility to DNA rearrangements, their large size and sensitivity to methylated DNA) (Falahi et al, 2013) have reduced their use in favour of other alternatives (ZFP and CRISPR-Cas9), and will not be considered in this Thesis.

4.2.1. Zinc finger proteins

Zinc fingers proteins (ZFP) are DNA-binding domains commonly found in eukaryotic transcription factors, in which each module of 30 aminoacids in a beta-beta-alpha structure recognizes 3bp of the DNA sequence (**Fig. 13**). Therefore, assembling six or more ZFP modules in tandem repeats allows unique specificity throughout the genome (Wolfe et al, 2000; Jurkowski et al, 2015).

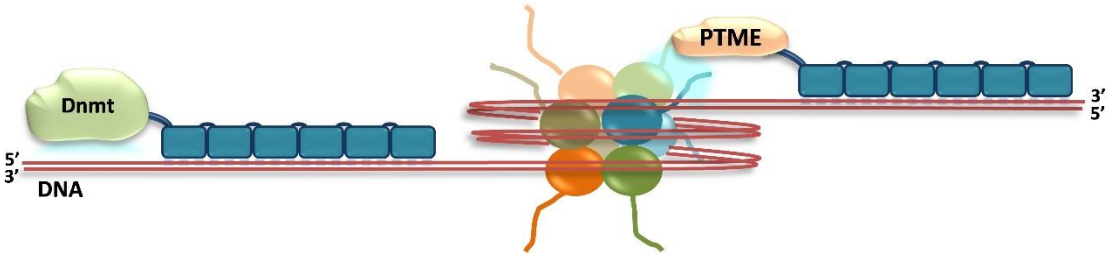


Figure 13. Targeted epigenetic editing with zinc finger proteins. Zinc finger proteins are composed of an array of modules, each one recognizing 3 nucleotides in the DNA sequence. Engineered Zinc finger proteins fused to different effectors (e.g. Dnmt, post-transcriptional modification enzymes) can target specific loci in the genome to add distinct chemical modifications (e.g. methylation on DNA or covalent modification on histone cues) (**Annex 1, Paper 4**).

Nunna and collaborators provided a brilliant example in cancer research, transfecting SOKV3 ovarian cancer cells with a chimeric ZF-Dnmt3a C terminal catalytic domain protein directed at the Epithelial Cell Adhesion Molecule (EpCAM) promoter (Nunna et al, 2014). EpCAM is overexpressed in several epithelial cancers, including ovarian, breast, pancreatic urothelial and gallbladder carcinoma and its expression levels inversely correlate with survival of patients (Schnell et al, 2013; Nunna et al, 2014).

Aberrant angiogenesis occurs in different pathological conditions, including endometriosis, being acknowledged as a hallmark of cancer (Hanaha and Weinberg, 2011; Guo, 2015). To this respect and by employing an engineered ZFP-Dnmt3a-Dnmt3L, Siddique and co-workers, successfully silenced the expression of vascular endothelial growth factor A (*VEGF-A*), the main regulator of angiogenesis, in an ovarian cancer SKOV3 cell line by methylating 12 CpGs at the *VEGF-A* promoter (Siddique et al, 2013). Similarly, Snowden and co-workers had decreased the expression of *VEGF-A* in HEK293 cells by specifically methylating H3K9 with specific ZF-SUV39H1/G9A constructs (Snowden et al, 2002).

The aforementioned studies exemplify the potential of ZFP for directing desired modifying enzymes to specific genomic loci. Nevertheless, the introduction of CRISPR-dCas9 technologies for epigenome editing has represented a revolution in the field, in part because of an easier design and the higher specificity of a RNA-DNA based interaction, rather than the protein-DNA based interaction provided by ZFP.

4.2.2. CRISPR/dCas9

CRISPR-Cas9 is considered an acquired immune system in bacteria and archaea (Mojica et al, 2005). These prokaryotes incorporate multiple foreign DNA sequences in a hypervariable region of its genome, the CRISPR locus. Subsequently, Cas nucleases incorporate cleaved transcripts from CRISPR loci and then scan the cytoplasm searching for foreign complementary sequences to be cleaved, in a RNAi-like mechanism of action (Brouns et al, 2008). For epigenetic editing purposes, engineered *Cas9* protein deficient in nuclease activity (dCas9), can be fused to a myriad of effector domains, expanding the epigenome editing tool repertoire. The mechanism of action is based on a short guide RNA (sgRNA) complementary to the target sequence directing the complex to the appropriate loci and requiring only an appropriate protospacer adjacent motif (PAM) sequence (e.g., 5'-NGG-3' for the *Streptococcus pyogenes*' Cas9) at the 3'-end of the targeted sequence for efficient binding and cleavage (Jurkowski et al, 2015) (**Fig. 14**).

As a proof of principle, Hilton and collaborators demonstrated that programmed dCas9 fused to the H3K27-acetylytansferase *p300* is a valid tool to promote gene expression throughout the genome both from promoters and from proximal and distal enhancers, as demonstrated with *IL1RN*, *MYOD1* and *OCT4* genes (Hilton et al, 2015). In another interesting approach, Thakore and co-workers targeted dCas9-KRAB to a distal HS2 enhancer, which regulates the expression of multiple globin genes. Expression of *HBE1*, *HBG1/2*, and *HBB* was reduced with minimal off-target effects (Takhore et al, 2015). Notably, KRAB has no catalytic activity but, instead, it recruits proteins to assemble a heterochromatin-forming complex that represses gene expression through histone methylation and deacetylation.

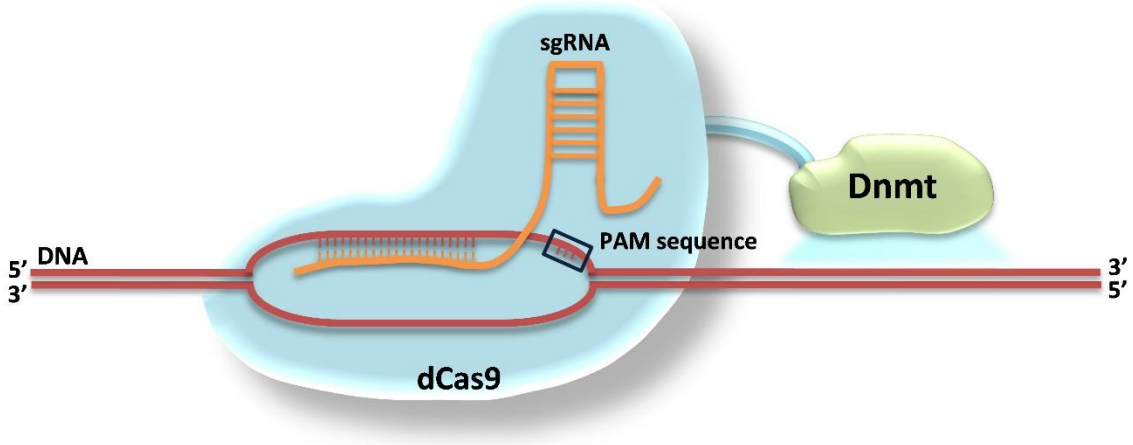


Figure 14. Targeted epigenetic editing with CRIPSR/dCas9 system. dCas9 nucleases recognize a specific locus in the DNA based on the Watson-Crick base pairing between a designed sgRNA and the genomic region of interest. An effector domain (e.g. *Dnmt3a*) introduces the desired chemical modification (cytosine methylation in this case). Dnmt: DNA methyltransferase; dCas9: deficient CRISPR-associated nuclease 9; sgRNA: single guide RNA; PAM: protospacer adjacent motif (**Annex 1, Paper 4**).

Vojta and collaborators published a seminal study in which methylation at specific promoters was achieved by using dCas9 fused to the catalytic domain of *DNMT3a* (Vojta et al, 2016). They specifically methylated and down-regulated the expression of both *BACH2* and *IL6ST* genes, which are involved in autoimmune diseases such as inflammatory

bowel disease. Shortly after, Stepper and collaborators achieved widespread methylation at the *EpCAM*, *CXCR4* and *TFRC* gene promoters both in HEK293 and ovarian cancer SKOV3 cells (Stepper et al, 2017). To do so, authors employed and engineered a dCas9-Dnmt3a-Dnmt3L construct. Conversely, methylation marks can also be removed with this engineered system, but in this case by tethering a *TET-1* DNA demethylase enzyme to the dCas9 protein (Choudhury et al, 2016; Xu et al, 2016).

Although CRISPR-dCas9 technology for epigenome editing is only taking its initial steps, the ease and robustness in targeting desired sequences, with tenuous off-target effects, assures great success in the epigenetic editing arena. In addition, this tool is multiplexable and several gRNAs can act in concordance to target the same or different genes at the same time (Du et al, 2016). Moreover, as *Cas9* derived from different prokaryotes require different PAM sequences, combination of different *Cas9* can broaden the spectrum of target genes (Jurkowski et al, 2015).

4.2.3. miRNA therapies: mimics and antimiRs

There are currently 479 clinical trials listed on microRNAs, the majority of them focusing on miRNA signatures for diagnosis and prognosis of a wide variety of diseases**.** (In MicroRNA clinical trials from Clinical Trials.gov. Accessed on October, 7th 2017 at http://clinicaltrials.gov/ct2/results?term=MICRORNA&Search=Search).

The goal in miRNA therapies is to restore the levels of miRNAs altered by the disease. Thus, whereas *antimiRs* target an upregulated miRNA in order to prevent its function, *miRNA mimics* are used to restore the physiological levels of a downregulated miRNA (reviewed in Gallach et al, 2014).

- For **antimiRs**, there are different therapeutic strategies for inhibiting endogenous miRNAs *in vivo* that are being evaluated in preclinical models, the simplest approach being an antisense RNA oligonucleotide (ASO) targeting the endogenous miRNA of interest (Anand, 2013). To increase the efficiency of antimir:miRNA pairing, several chemical modifications at the ribose are incorporated, the most commonly used rendering a 2'-fluoro (2'-F), 2'-O-methoxyethyl (2'-MOE) and 2'-O-methyl (2'-O-Me) ribose, a morpholine ring replacing the sugar in morpholino oligomers and a sulphur replacing one of the non-bridging oxygen atoms in the phosphate group in oligomers with phosphorothioate (PS) linkage. An additional modification is performed by locking the ribose in a C3'-endo conformation by introduction of a 2'-O,4'-C methylene bridge. This renders a Locked Nucleic Acid (LNA), which is a bicyclic RNA analogue (Stenvang et al, 2012). Interestingly, this chemical modification is also implemented in primers for PCR reactions, being the chemistry we have chosen for the experiments showed in this work, as will be described in Material and Methods section. Additionally, **miRNA sponges** (a synthetic mRNA with binding sites for multiple endogenous miRNA) and **miR-Mask** (a 2'-O-methyl-modified synthetic miRNA complementary to the miRNA binding site in the 3'-UTR of the mRNA of interest) are also being tested (Gallach et al, 2014).

- Conversely, **miRNA mimics** are based on the use of double-stranded synthetic oligonucleotides that mimic endogenous miRNAs and are processed into the cell when transfected (Gallach et al, 2014).

Although progresses are required to efficiently deliver miRNA-based-therapeutic agents to the target tissue and diminish off-target effects, in some pathologies these therapies are promising. For hepatitis C virus infection, an AntimiR-122 called Miravirsen® is being tested in several ongoing Phase II clinical trials (ClinicalTrials.gov identifiers: NCT02031133, NCT01727934, NCT02508090, and NCT01872936), and a mimic of the tumour suppressor miR-34 named MRX34® is being tested in an ongoing phase I multicentre clinical trial for patients of hepatocellular carcinoma and other selected solid tumours or haematological malignancies (ClinicalTrials.gov identifier: NCT01829971).

Regarding endometriosis, the only clinical trial mentioning the disease is entitled "Clinical Validation of the Role of microRNA Binding Site Mutations in Cancer Risk, Prevention and Treatment", considering as a study cohort women with drug resistant endometriosis. This lack of clinical trials could be explained by the lack of an optimal miRNA candidate suitable for therapeutic development. Instead, great research endeavours are conducted to overcome this limitation, of which an example is provided in the Chapter 3 of the present work.

II. Hypothesis and objectives

II. Hypothesis and objectives

1. Hypothesis

Endometriosis is a multifactorial disease, in which endometrial and peritoneal factors may be involved. Furthermore, several authors have pointed to altered epigenetic mechanisms as important players in the aetiology and pathogenesis of this condition.

Thus, our **overall hypothesis** is that an aberrant epigenetic profile could modulate the molecular mechanisms underlying the aetiology and pathophysiology of endometriosis. This hypothesis is developed in the following specific hypothesis:

1. Our **first hypothesis** is that endometrial factors, in terms of an aberrant miRNA and DNA methylation profile, are responsible for the altered survival, proliferation, angiogenesis and extracellular matrix remodelling observed in the disease.

2. Our **second hypothesis** is that peritoneal factors, in terms of altered composition of peritoneal fluid in patients with endometriosis, could mediate the deregulation of miRNAs in endometrial and endometriotic stromal cells, being responsible for the altered biological processes observed in this pathology.

3. Finally, our **third hypothesis** is that the expression of VEGF-A in stromal cell cultures can be epigenetically modulated by means of selected miRNA mimics and of targeted DNA methylation at *VEGFA* promoter.

2. Objectives

1. Our **first objective** was to assess miRNA expression profiles in samples of eutopic and control endometria and ovarian endometrioma from women with endometriosis; and also, to validate the results in a higher cohort of samples, including paired peritoneal implants and rectovaginal nodules. Additionally, we aimed to evaluate the role of deregulated miRNAs on the expression of the main regulators of angiogenesis and fibrinolysis.

2. Our **second objective** was to validate our *in vitro* model in primary stromal cell cultures from endometrial samples and from ovarian endometrioma. Additionally, we wanted to evaluate the influence of peritoneal fluid on the expression of selected angiogenesis- and fibrinolysis-related miRNAs.

3. Our **third objective** was to assess the influence of peritoneal fluid on miRNA expression profiles in primary endometrial and endometriotic stromal cell cultures. Additionally, we wanted to validate *VEGFA* mRNA as a target of miR-16-5p, miR-29c-3p, miR-424-5p.

4. Our **fourth objective** was to define the DNA methylation profiles in endometrial samples from patients with endometriosis and from control women by employing the Illumina Infinium MethylationEPIC BeadChip.

5. Our **fifth objective** was to down-regulate the expression of VEGF-A in primary stromal cell cultures of patients with endometriosis by employing techniques for targeted DNA methylation (fused Zinc fingers proteins – Dnmt3a3L enzyme).

III. Materials and Methods

III. Materials and Methods

1. Clinical groups

Women included in this study were recruited in the gynaecological services of the Hospital Universitario y Politécnico La Fe (Valencia) and Hospital General Universitario (Valencia).

For the **endometriosis cohort**, the diagnosis was performed by macroscopic assessment during a laparoscopic surgery of the entire pelvic anatomy and the rest of the abdominal cavity. Characteristics of the adnexal cyst and its content were exhaustively analysed when present. A careful search for peritoneal implants, atypical lesions, peritoneal defects, or adhesions was performed to provide a correct staging of the disease, performing a biopsy of any suspicious area. Subsequently, a pathological examination was conducted to evaluate the presence of endometrial glands and stroma in the extracted samples and confirm the diagnosis of endometriosis. Women with suspected endometriosis but without anatomopathological confirmation of the disease were excluded from the study. Only women in stages III-IV were included.

Women considered as **control cohort** consisted of fertile women without endometriosis, who underwent surgery for laparoscopic tubal sterilization. The absence of endometriosis was confirmed by meticulous examination of the pelvic and extrapelvic peritoneum, ovaries, intestine, and diaphragm confirming the absence of typical or atypical endometriotic lesions. Biopsies of suspicious areas for endometriosis were confirmed to be negative in these women.

All participants signed the informed consent. The research was performed following the ethical principles of the Declaration of Helsinki and its successive amendments (World Medical Association, 2013). The study was approved by the Ethical Committee for Biomedical Research of the Hospital Universitario y Politécnico La Fe (2008/0111; 2011/01194) and Hospital General Universitario of Valencia (CEIC-CHGUV: 30-10-2014).

1.1. Inclusion criteria

All women recruited for the study were adult (at least 18 years old), pre-menopausal and with regular menstruation. As an indispensable requisite, all of them signed the informed consent and neither underwent any hormonal treatment nor had been breast-feeding for six months prior to study.

1.2. Exclusion criteria

For the accomplishment of the study, those patients with irregular menstruation (menorrhagia), those who were pregnant or who had been breast-feeding in the 6 months prior to the study or with hormonal treatment in the 6 months prior to the study were excluded.

1.3. Analysis of the phase of the menstrual cycle

The phase of the menstrual cycle was determined according to following criteria: 1) Self-reported day of the reproductive cycle and 2) Histological analysis of the endometrium following Noyes criteria (Noyes et al, 1975).

2. Type of samples per cohort of study

2.1. Endometrial and endometriotic tissue biopsies

Tissue samples from women with endometriosis consisted of biopsies of endometrium (eutopic endometrium, EUT) and of endometriotic lesions: ovarian endometriomas (OMAs), peritoneal implant (PI) and nodule in the Pouch of Douglas or rectovaginal nodule (RVN).

Tissue samples from women without endometriosis consisted of biopsies of endometrium (control endometrium, CNT).

OMA specimens were obtained with Pipelle® endometrial biopsy sampler from the capsule of the endometriotic cyst, extracted in the course of conservative surgery performed by laparoscopy. Endometrial tissues were obtained by endometrial biopsy-aspiration during anaesthetic induction in both endometriosis patients and control women.

All tissues were collected in Corning tubes containing PBS, 50U penicillin/mL and 50 µg/mL streptomycin (Sigma) and washed with cold PBS buffer to remove blood cells. For the extraction of RNA, DNA and protein, a partition of the tissue was frozen in liquid nitrogen and stored until processing. To verify the diagnosis and the phase of the cycle, another part of the tissue was fixed overnight at 4°C and embedded in paraffin blocks for anatomopathological studies (Pathology Departments of Hospital Universitario y Politécnico La Fe and Hospital General Universitario of Valencia, when corresponding).

2.2. Peritoneal fluid

Peritoneal fluid (PF) samples were collected from the bottom of the pouch of Douglas and from the vesico-uterine space of both patients and control women. No peritoneal lavage was performed prior to sample collection and no anticoagulant was used. The fluid was collected in vacuum tubes with a sterile syringe, which was attached to an endoscopic catheter in the laparoscopic approach. Liquids with blood contamination were excluded. PFs were centrifuged at 3000 rpm for 30 minutes at 4 °C in a refrigerated centrifuge (P-SELECTA) in order to be cleared from cells and debris. Subsequently, PFs were sterilized by filtration through 0.22 µm filters (Merck) in a laminar flow cabinet (Steril-Helios) under sterile conditions. Supernatant were stored in aliquots at -80 °C.

3. Participants per study

3.1. Clinical groups for tissue miRNA studies (Chapter 1)

- *Endometriosis cohort*

51 Caucasian women with endometriosis (stage III-IV) were studied (mean age: 34.0 years, range: 20–45). The main symptom for surgery in this group of patients was abdominal pain (74.5%) and sterility (25.5%). 51 paired EUT and OMAs, together with 18 PIs and 20 RVNs were analysed in these patients. 26 (51%) women were in the proliferative phase and 25 (49%) in the secretory phase of the menstrual cycle. Menstrual phase was excluded from the study.

- *Non-endometriosis control cohort*

32 Caucasian asymptomatic women without endometriosis, who underwent surgery for laparoscopic tubal sterilization, were included in the control group (mean age: 36.4 years, range: 27–45). 32 CNT were analysed in these patients. 15 (47%) women were in the proliferative phase and 17 (53%) were in the secretory phase of the menstrual cycle. Women in the menstrual phase were excluded from the study.

3.2. Clinical groups for *in vitro* studies (Chapter 2 and Chapter 3)

- *Tissue samples for primary stromal cell cultures*

In order to isolate stromal cells, 11 EUT tissues (eutopic cells) (mean age 32 years; range 19–40) and 11 OMAs (ovarian endometrioma cells) from women with moderate or severe endometriosis (stage III-IV) (mean age 30 years; range 19–42) and CNT tissue (control cells) from 8 women without the disease (mean age 36 years; range 24–43) were obtained.

- *Peritoneal fluid pools*

To stimulate primary stromal cell cultures, 10 PFs from women with endometriosis (endometriotic peritoneal fluid pool, EPF) (mean age 33.1 years, range 27-39) and 10 PFs from fertile women without endometriosis (control peritoneal fluid, CPF) (mean age 37.2, range 21-47) in the proliferative phase of the menstrual cycle were thawed and pooled.

3.3. Clinical groups for DNA methylation studies (Chapter 4)

- *Tissue samples for DNA methylation profiles*
    - *Endometriosis cohort*

13 Caucasian women with endometriosis (stage III-IV) were included in the study (mean age 35.2 years, range 24-42). 5 (38.4%) women were in the proliferative phase, 4 (30.8%) in the secretory phase and 4 (30.8%) in the menstrual phase of the cycle. Regarding endometriotic lesions, OMAs was a common lesion for all participants. Of note, only two patients had one additional endometriotic lesion, either PIs or RVNs, respectively.

- *Non-endometriosis cohort*

11 Caucasian women without endometriosis were included in the study (mean age 36.6 years, range 30-44). 4 (36.4%) women were in the proliferative phase, 4 (36.4%) in the secretory phase and 4 (27.2%) in the menstrual phase of the cycle.

3.4. Clinical group for epigenetic editing (Chapter 5)

In order to perform primary stromal cell cultures, 3 endometrial tissues (eutopic cells) (mean age 36 years; range 30-40) from women with moderate-severe endometriosis (stages III and IV) and 2 endometrial tissues (control cells) from women without the disease (control cells) (mean age 35.5 years; range 34-37) were obtained. Notably, tissues from one patient with endometriosis and from the two control women had also been analysed in Chapter 5.

4. Cell cultures

4.1. Primary cell cultures of endometrial and endometriotic stromal cells

A fragment of endometrial biopsies and OMAs tissues were cut into 1 mm^3 fragments and incubated at 37 °C during 60' in phenol-red free DMEM (Thermofisher Scientific) in the presence of 2.5 mg/mL of collagenase (Sigma) for extracellular matrix digestion. Subsequently, digested tissues were filtered through a 70 μm filter (BD Falcon) to remove undigested material and larger epithelial cells. The cell suspension was then centrifuged at 550xg for 5' and the cell pellet was resuspended in DMEM-F12 medium (Thermofisher Scientific) without phenol red, supplemented with 10% FBS (Invitrogen), 50 U/mL penicillin and 50 μg/mL streptomycin (Sigma). Cell viability, calculated by the trypan blue exclusion test (Sigma), was found to be greater than 95%. Under these conditions, stromal cells were cultured in 25 cm^2 flasks, keeping the atmosphere humidified to 5% CO_2 and 95% air at 37 °C. The culture medium was renewed every 2 days until the cell monolayer reached 80% of confluence, proceeding then to subculturing. For this purpose, cells were detached with 0.25% trypsin/ 0.02% EDTA (Gibco BRL) at 37 °C, and seeded at a density of $2.7x10^5$ cells/well in 12-well plates. The purity of the endometrial stromal cell suspension was determined by positive staining for vimentin (Abcam) (stromal and epithelial cells) and negative cell staining for both cytokeratin (Abcam) (epithelial cells) and CD68 (Abcam) (macrophages), the result being higher than 95%.

4.2. Commercial Cell lines

The endothelial cell (EC) line EA.hy926 was obtained from the American Type Culture Collection (ATCC). ECs were maintained in phenol-red free DMEM supplemented with 2 mM glutamine and 10% FBS (Life Technologies).

A human colon cancer cell line HCT-116 deficient for Dicer (HCT-DK) was a kind gift from Dr. Renato Baserga (Thomas Jefferson University, USA). HCT-DK were cultured in McCoy's 5A (Sigma-Aldrich) supplemented with 2 mM glutamine and 10% FBS (Life Technologies).

Finally, HEK-293 cells (ATCC) were maintained at 37 ºC with 5% CO2 in DMEM media (Thermofisher Scientific) supplemented with 10% FBS (Invitrogen), 50 U/mL penicillin and 50 μg/mL streptomycin (Sigma).

5. Basic laboratory techniques

5.1. Techniques for nucleic acid extraction

5.1.1. Total RNA extraction

Total RNA, including the fraction of miRNAs, was extracted from endometrial and endometriotic tissues (Chapter 1) and stromal cell cultures (Chapter 2 and Chapter 3) using the column based kit "mirVana miRNA isolation kit" (Ambion) following manufacturer's recommendations.

For epigenetic purposes (Chapter 5), RNA was extracted with RNeasy® Plus Mini Kit (Qiagen), following manufacturer's recommendations. In the final step, RNA was eluted to a Vf = 30 μL.

5.1.2. DNA extraction

Genomic DNA was extracted from EUT and CNT tissues with the DNeasy® Blood & Tissue Kit (QIAGEN), following manufacturer's recommendations.

Tissues were cut tissue into small pieces of 25 mg, and placed in a 1.5 mL microcentrifuge tube, containing 180 μL of buffer ATL. After addition of 20 μL of proteinase K and mix by vortexing, samples were incubated at 56°C for 1h. After incubation, samples were vortexed during 15 s before adding 200 μL of Buffer AL and add 200 μL ethanol (96–100%). Mixture was pipetted into an appropriate spin column placed in a 2 mL collection tube, and centrifuge at 6000g for 1 min. After several serial washing steps with 500 μL Buffer AW1 and 500 μL Buffer AW2 (twice for the latter) for 1 min at 6000 g, spin columns were placed into a new 2 mL collection tube, centrifuged for 3 min at 20,000 g and, after transferring the spin column to a new 1.5 mL microcentrifuge tube, DNA was eluted by adding 200 μL Buffer AE to the centre of the spin column membrane. Elution was performed after incubating for 1 min at 25°C and centrifuging for 1 min at 6000 g.

To extract DNA for epigenetic editing purposes, trypsinized were pelleted in a 1.5 mL Eppendorf tube and resuspended with 200 μL of PBS. Then, we employed the QIAamp DNA Mini Kit (Qiagen), following manufacturer's recommendations. The final elution step was performed with 25 μL of AE buffer.

5.2. Techniques for protein quantification

5.2.1. Protein extraction

Cytosolic and membrane protein extracts from endometriotic and endometrial tissues were obtained as previously described (Bouchet-Bernet et al, 1996) with minor modifications. Briefly, tissues were immersed in 1.2 mL of **buffer A**, adding 12 μL of protease inhibitor buffer. Thereafter, tissues were mechanically disrupted on ice with an

ultra-turrax™ T-8 disperser (IKA) in three runs of 10 s each. Thus, a lysate was obtained with a mixture of enzymes, membranes and broken cells. This preparation was subjected to ultracentrifugation for 15 min at 35,000 rpm in a CP100NX ultracentrifuge (Hitachi), to separate the supernatant (containing the cytosolic proteins) from the precipitate (containing cell debris and membrane associated proteins). To extract the membrane-associated proteins present in the precipitate, 1.2 mL of Buffer B were added to the pellet. Finally, they were centrifuged at 35,000 g for 15 min in a CP100NX ultracentrifuge (Hitachi), obtaining the total proteins in the supernatant. These fractions were aliquoted and stored at -80° C until processing.

5.2.1.1. Buffers for total protein extraction

- Protease inhibitor buffer: HEPES (10 mM), EDTA (1 mM), EGTA (1 mM), KCl (10 mM), DDT (1 mM), NaF (5 mM), Na_2VO_4 (1 mM), Na_2MoO_4 (10 mM), leupeptin (1 μg/mL), aprotinin (0,1 μg/mL), phenylmethylsulfonylfluoride (0.5 mM), adjusted at pH=8.

- Buffer A: Tris-HCl (10 mM), dithiothreitol (0.5 mM), EDTA·Na_2 (1.5mM), adjusted at pH=7,4 and then: glycerol (10%) and distilled H_2O until completing a volume of 100 mL

- Buffer B: Tris-HCl (20 mM), NaCl (125 mM), adjusted at pH 7.4 and then: triton X-100 (1%) and distilled H_2O until completing a volume of 100 mL.

5.2.2. Total protein quantification: BCA assay

In order to normalize the antigenic levels obtained in the protein extracts, the amount of total protein in the samples was determined using the Pierce BCA Protein Assay (Thermo Scientific), following the manufacturer's instructions. Samples were quantified in duplicate.

5.2.3. Western Blot

VEGF-A protein expression from cells lysates (Chapter 2 and Chapter 3) was quantified by western blot. The employed anti-VEGF antibody (Abcam) recognizes both human VEGF-A_{165} and VEGF-A_{121} isoforms.

5.2.4. Enzyme-Linked Immunosorbent Assay (ELISA)

VEGF-A, TSP-1, uPA and PAI-1 protein levels were determined by ELISA in cell culture supernatants, PF pools and endometrial and endometriotic tissue lysates. For cell culture supernatants, the protein amounts released to the culture medium by cells incubated with PF pools were calculated by subtracting VEGF-A, uPA, TSP-1and PAI-1 contents in the PF pool to the total levels obtained in supernatants.

- *VEGF-A*

VEGF-A protein level was measured by using a commercially available ELISA (IBL International), following manufacturer's recommendations. No cross-reactivity or interference with platelet derived growth factor was observed. This assay recognizes human VEGF-A_{165} and VEGF-A_{121} isoforms. The intra-assay and inter-assay variation coefficients were 4–6% and 7–10%, respectively.

- *TSP-1*

TSP-1 protein levels were quantified using a commercially available ELISA (R&D Systems), following manufacturer's recommendations. No cross-reactivity or interference with TSP-2 or TSP-4 was observed. The intra-assay and inter-assay variation coefficients were 5–6% and 8–11%, respectively.

- *uPA*

uPA protein levels were quantified by a commercially available ELISA (Hyphen Biomed), following manufacturer's recommendations. This kit allows the measurement of single-chain urokinase (scuPA) and the high weight molecular form of uPA (HMW-uPA) with similar efficiency. The intra-assay and inter-assay variation coefficients were 3–5% and 8–11%, respectively.

- *PAI-1*

PAI-1 protein levels were quantified by a commercially available ELISA (America Diagnostica), following manufacturer's recommendations. The assay detects free and complexed PAI-1 and is insensitive to PAI-2. The intra-assay and inter-assay variation coefficients were 3–4% and 6–8%, respectively.

6. Epigenomic techniques for nucleic acid profiling

6.1. Spectrophotometric determinations

Yield and purity of the extracted DNA and RNA were determined using a NanoDrop ND-1000 spectrophotometer (Thermofisher Scientific). For RNA studies, only samples with total RNA concentration >100 ng/µL, and absorption ratios A_{260}/A_{280} and A_{260}/A_{230}> 1.8 were considered suitable for subsequent analyses (according to the manufacturer's recommendations).

6.2. miRNA expression microarrays

6.2.1 Agilent 2100 bioanalyzer

RNA integrity was analyzed using the Agilent 2100 Bioanalyzer (Agilent Technologies). All samples employed for microarray assays presented a RNA integrity number ≥9.0. based on Ibberson's definition of good quality RNA (Ibberson et al, 2009).

6.2.2. Affymetrix GeneChip miRNA 2.0 arrays

miRNA expression microarrays were performed in total RNA extracted from endometriotic and endometrial tissues (Chapter 1) and cell cultures (Chapter 3) with the GeneChip miRNA 2.0 arrays (Affymetrix), in the Array Service of our institution. For each sample, 500 ng of total RNA were labelled with the FlashTag Biotin RNA Labelling Kit (Genisphere) according to manufacturer's recommendations. Briefly, a tailing reaction was carried out at 37 ºC for 15 min (10 mL of total RNA and spike control oligos mix, 1× reaction buffer, 2.5 mM $MnCl_2$, 1.33 mM ATP and 1 ml PolyA polymerase enzyme), and this was followed by ligation of the biotinylated signal molecule to the target RNA sample at 25 ºC for 30 min (with the addition of 4 mL of 5×Flash-Tag Ligation Mix Biotin and 2 mL of T4 DNA Ligase into the 15 mL of reaction mix). Finally, 2.5 mL of stop solution were added to terminate the reaction. Labelled RNAs were hybridized on GeneChip miRNA 2.0 arrays (Affymetrix) at 48 ºC and 60 rpm for 16 h in the presence of total biotin-labelled RNA, hybridization mix, formamide, DMSO, eukaryotic hybridization controls and control oligonucleotide B2. Immediately following hybridization, the arrays were washed and stained with streptavidin–phycoerythrin conjugate in the GeneChip Fluidics Station 450 (Affymetrix). Finally, they were scanned using a GeneChip Scanner 3000 7G (Affymetrix).

6.3. DNA methylation profiles

6.3.1. PicoGreen

All DNA samples were treated with RNase A for 1 h at 45 ºC and quantified by the fluorometric method Quant-iT Pico Green dsDNA Assay (Life Technologies), following manufacturer's recommendations.

6.3.2. DNA HumanMethylationEPIC BeadChip

Epigenomic studies were performed in the Epigenomics core facility of our institution. We employed the Infinium HumanMethylationEPIC BeadChip platform (Illumina) to interrogate over 850,000 CpG sites, which has been previously established as a reliable

technology to detect epigenetic alterations in terms of DNA methylation (Moran et al, 2016).

600 ng of purified DNA were randomly distributed on a 96-well plate, and processed using the EZ-96 DNA Methylation kit (Zymo Research Corp.) following the manufacturer's recommendations for Infinium assays. Bisulphite-converted DNA (bs-DNA) was processed as previously described (Sandoval et al, 2011). MethylationEPIC BeadChip array shares the Infinium HD chemistry Assay (Illumina) used to interrogate the cytosine markers with previous releases, as the HumanMethylation450k BeadChip (Illumina). Thus, the applicable protocol for MethylationEPIC is the same as for HumanMethylation450k, which is the Infinium HD Methylation Assay Protocol. Briefly, 4 µL of bisulphite-treated tissue DNA were processed following this protocol, as previously described (Sandoval et al, 2011). The resulting raw data (IDATs) was imported into R using the minfi package (version 1.22.1) (Bioconductor) and functional normalization was applied (control normalization). Background signal was corrected using the methylation module (1.9.0) available on GenomeStudio (v2011.1) software (Illumina). CpGs with detection values lower than 0.01 were removed from the array. Additionally, CpGs of the X and Y chromosomes were also deleted, as well as the CpGs with "ch" or "rs" in the name. From all these filters, the final β-values were calculated, which served as a variable for all statistical analyses. After filtering according to the above criteria, 866,836 CpGs corresponding to 23 samples were considered for statistical analyses (See point 9.2 of this section). These CpG markers present on MethylationEPIC BeadChip are classified based on: 1) their chromosome location, 2) the Infinium chemistry used to interrogate the marker (Infinium I, Infinium II) and 3) the feature category gene region as per UCSC annotation (TSS200, TSS1500, 5′-UTR, 1st Exon, Body, 3′-UTR). Additional criteria included the location of the marker relative to the CpG island (open sea, island, shore, shelf), fantom 5-associated enhancer regions and regulatory regions described on ENCODE project (Sieggens and Ekwall, 2014; Lizio et al, 2015) such as transcription binding site sequences, open chromatin regions and digital DNase I hypersensitivity clusters. These analyses and data treatment were performed at the Epigenomics core facility from our institution whose director is Dr. Sandoval, one of my thesis directors.

7. Epigenetic techniques for nucleic acid quantification

7.1. RNA

7.1.1. mRNA quantification by qRT-PCR

To analyse the mRNA expression levels of VEGF-A (*VEGFA*), TSP-1 (*THBS1*), uPA (*PLAU*) and PAI-1 (*SERPINE1*) (Chapter 2), 1 µg of total RNA was treated with DNase I (Invitrogen) and reverse transcribed into first-strand cDNA by using Superscript RNase H- (Invitrogen) with an oligo$(dT)_{15}$ primer (Promega). cDNA was stored at -20ºC until subsequent study. Additionally, β-actin (*ACTB*) mRNA was quantified for normalization purposes. Polymerase Chain Reaction (PCR) was performed in a Light Cycler 480 II thermocycler, using version 3.5 software (Roche Molecular Biochemicals).

The specific primers used for amplification of the aforementioned genes were designed using the software Primer3 (http://bioinfo.ut.ee/primer3-0.4.0/) (**Table 1**). These

sequences were analysed by FASTA in the EMBL database (http://www.embl-heidelberg.de/).

Table 1: Oligonucleotide PCR primers for mRNA amplification (Chapter 2)

Gene name	Oligonucleotide primer sequence	Fragment size (bp)
PLAU		
Forward primer	5'-cacgcaaggggagatgaa-3'	341
Reverse primer	5'-acagcattttggtggtgactt-3'	
SERPINE1		
Forward primer	5'-tgctggtgaatgccctctact-3'	399
Reverse primer	5'-cggtcattcccaggttctcta-3'	
VEGFA		
Forward primer	5'-atcacgaagtggtgaagttc-3'	265
Reverse primer	5'-tgctgtaggaagctcatctc-3'	
THBS1		
Forward primer	5'-tgtgaaaagatggagaatgctg-3'	275
Reverse primer	5'-ttgtggccaatgtagttagtgc-3'	
ACTB		
Forward primer	5'-cgtaccactggcatcgtgat-3'	452
Reverse primer	5'-gtgttggcgtacaggtctttg-3'	

Each assay was carried out in a final volume of 10 µL containing 1.5 µL cDNA (1:10), 3 mM $MgCl_2$, 0.5 mM of each VEGF-A/TSP-1/uPA/PAI-1 primer or 0.3 mM of β-actin primer, 1 µL LC-Fast Start Reaction Mix SYBR Green I and 1 µL LC- Fast Start DNA Master SYBR Green I/Enzyme, including TaqDNA polymerase, reaction buffer and desoxynucleotide triphosphate mix (Roche Molecular Biochemicals). The amplification program consisted of the following three steps: 1) The first step was an initial heating for 10 min at 95 ºC to denature the cDNA and to activate the TaqDNA polymerase. Then, 2) DNA was amplified for 40 cycles of 15s at 95 ºC for β-actin, uPA and PAI-1 or 0 s for VEGF-A and TSP-1 (denaturation), 5 s at 60 ºC for uPA and PAI-1 or at 62 ºC for β-actin or 10s at 60 ºC for VEGF-A or 62 ºC for TSP-1 (annealing) and 18 s at 72ºC for β-actin, uPA, PAI-1 or 12s for TSP-1 or 10 s for VEGF-A (extension). Finally, 3) the temperature was raised gradually (0,18 ºC/s) from 65 ºC to 95 ºC for the melting curve analysis. To verify the melting curve results, representative samples of the PCR products were assayed on 2% agarose gels. Two negative controls were included in each assay: one without a template sample and another one without reverse transcriptase. The number of sample copies was calculated by setting their crossing points to the standard curve. Data are showed as the ratio target cDNA concentration/β-actin cDNA concentration.

To analyse the mRNA expression levels of VEGF-A (Chapter 2), 400 ng of total RNA and Super-Script™ III First-Strand Synthesis System (Life Technologies) were used for reverse transcription reactions, following manufacturer's recommendations. VEGFA and β-actin (*ACTB*) (as endogenous reference control) gene expression was quantified by PCR. (Probe references: Hs00900055_m1 and Hs99999903_m1, respectively; Life Technologies).

For epigenetic editing purposes (Chapter 5), *VEGFA* mRNA expression levels were determined as follows: for cDNA synthesis, 500 ng of total RNA at a final volume of 16.25 µL were retrotranscribed in a two-step protocol. First, 1.25 µL of dNTPs (10 mM) and 2.5 µL of oligodT were incubated 2 min at 25 ºC. Afterwards, 2.5 µL of 10X M-MuLV RT buffer (New England Biolabs), 1.25 µL of M-MuLV reverse transcriptase enzyme (New England Biolabs) and 1.25 µL (40 U/µL) of RNAse Inhibitor were added

to the product of the first reaction and incubated for 2 h at 42 ºC, followed by an enzyme-inactivation step for 30 min at 65 ºC. Subsequently, each PCR reaction consisted of 1 µL of cDNA, 7.5 µL of SsoFastEvaGreen supermix, 0.4 µL of forward and reverse primer (500 nM, see **Table 2**), and 5.7 µL of nuclease-free H_2O. Experiments were performed in a CFX96 instrument (BioRad), following and optimized program consisting of 30 s at 95 ºC (1 cycle), 5 s at 95ºC plus 5 s at 60 ºC (40 cycles). Then, a melting curve was performed (60-95 ºC, rate = 0.5 ºC/2 s). Amplicons were confirmed by melting analysis. No template and no RT controls were included in all sets, and they always gave signal only in very late cycles. The amount of the *VEGFA* mRNA was quantified and normalized to the control gene *ACTB*.

Table 2: Primers used for *VEGFA* and *ACTB* mRNA qRT-PCR in stromal cell cultures (Chapter 5).

VEGFA _Forward:	5'- agaaggaggagggcagaatca - 3'
VEGFA _Reverse:	5'- atggcttgaagatgtactcg - 3'
ACTB_Forward:	5'- cgtaccactggcatcgtgat - 3'
ACTB_Reverse:	5'- gtgttggcgtacaggtctttg - 3'

In all cases, the $2^{-\Delta\Delta Ct}$ method was followed to calculate the relative abundance of mRNA compared with endogenous control expression (Ct= threshold cycle; ΔCt= Ct endogenous control— Ct sample gene).

7.1.2. Mature miRNA quantification by qRT-PCR

Quantification of mature miRNAs selected for validation was performed by means of quantitative real time PCR (qRT-PCR), using the miRCURY LNA™ Universal RT microRNA PCR kit (Exiqon). For normalization purposes, the small nuclear RNA U6 (*RNU6A*) served as endogenous "housekeeping" gene.

The miRCURY LNA™ Universal RT microRNA PCR (Exiqon) is based on a single universal retrotranscription (RT), followed by a real-time amplification by PCR with chemically modified primers (LNA™, see Stenvang et al, 2012 for further details about LNA™ nucleotides chemistry).

The protocol was performed as detailed: PCR reactions were carried out in a Light Cycler II 480 thermal cycler (Roche). Steps for PCR included an initial polymerase activation/denaturation at 95 ºC for 10 min, followed by 45 cycles of amplification, each of which included 10 s of heating at 95 ºC, and 1 min at 60 ºC with a ramp-rate of 1.6 ºC/s both for cooling from 95 ºC and for heating from 60 ºC until 95 ºC. To discriminate between specific and unspecific Sybr Green signal, a melting curve was performed for all the samples analysed, following a heating step from 60 ºC to 95 ºC at a ramp-rate of 0,11 ºC/s and a cooling to 4 ºC after the 45th cycle of amplification. All samples were analysed in duplicate.

7.2. DNA

7.2.1. Bisulphite sequencing – DNA methylation analysis at single CpG level

The sequencing of bisulphite converted DNA obtained from eutopic stromal cells requires a protocol 8-step protocol, hereafter described:

1. *DNA digestion*: to allow a better performance of the bisulphite conversion reaction, 300 ng of extracted DNA were digested with 2 µL of 2 buffer N.3. (Qiagen), 2 µL (40 U) of Bam HI nuclease (Sigma Aldrich) and 10 µL of nuclease-free H_2O to a final volume of 20 µL. The reaction mixture was O/N incubated at 37ºC.

2. Bisulphite conversion: 20 µL of digested DNA were bisulphite converted using EZ DNA Methylation-Lightning™ Kit (Zymo Research), following manufacturer's recommendations. Elution was achieved with 20 µL of M-Elution Buffer. Then, the reaction product was kept on ice until the next step was performed.

3. VEGFA PCR: the bisulphite converted DNA (bs-DNA) was used for PCR amplification using the amplicon, specific primers and an optimized HotStarTaq polymerase. Briefly, the reaction mixture consisted of 3 µL of bs-DNA, 12.1 µL of nuclease-free H_2O, 2 µL of 10x E-buffer, 200 µM of dNTPs, 0.5 µL of SuperHot polymerase (Genaxxon bioscience), and 0.5 µL of both forward and reverse primers (**Table 3**) (Vf =20 µL). This mixture was incubated with a specific PCR program: 1 cycle of 10 min at 95 ºC, 40 cycles of 30 s at 94 ºC, 30 s at 47 ºC and 1 min at 72 ºC; followed by a step of 10 min at 72 ºC. Then, the PCR product was cooled-down and hold at 8 ºC.

Table 3: Primers used to amplify *VEGFA* for bisulphite sequencing (Chapter 5).

VEGFA _Forward:	5'-gctcggtgctgg aatttgat-3'
VEGFA _Reverse:	5'-gcgagaacagcccagaagtt-3'

4. *Electrophoresis of PCR products:* 20 µL of PCR products and 4 µL of loading buffer (Thermofisher Scientific) were run in a 1% agarose (Biozym)-TPE-Gel red (1: 25,000 dilution; Phoenix research) gel. Electrophoresis conditions: 80 V, 60 min.

5. *Purification of VEGFA amplicon from agarose gel.* The fragment corresponding to the *VEGFA* amplicon was cut after identification under a UV lamp and placed into an Eppendorf tube with 2 µL/mg of gel of NTC (NucleoSpin® Gel and PCR Clean-up; Macherey-Nagel). After heating at 50 ºC for 5 min, DNA extraction was performed following manufacturer's recommendations, in a final volume of 15 µL.

6. *End A labelling:* purified *VEGFA* amplicon was labelled with Adenine at the 3'-ends. To this end, a reaction mixture consisting of 15 µL of PCR fragment, 278 µM of dNTPS, 2 µL of 10X Pfu buffer (Promega) and 0.5 µL of Taq polymerase (Genaxxon bioscience) (Vf =18 µL). The reaction mixture was incubated for 15 min at 72 ºC.

7. *TOPO® TA cloning*. Cloning is done using TOPO® TA cloning® kit (Life Technologies). For this, 2 µL of End A-labelled DNA, 0.5 µL of salt solution and 0.5 µL of TOPO® TA vector were incubated for 10 min at RT. Afterwards, 1 µL of the reaction product were added to 25 µL of competent *E.coli* cells, avoiding pipetting to mix. After

30 min of incubation on ice, bacteria were heat shock transfected (30 s at 42 ºC). Tubes were immediately transferred on ice. Then, 125 µL of RT SOC medium (Thermofisher Scientific) were added and tubes were incubated at 37 ºC for 1h in horizontal position. Total volume was seeded on pre-warmed (37 ºC) agar plates containing ampicillin and 2% X-gal. Colonies were grown O/N at 37 ºC. Under these conditions, positive colonies for the amplicon-containing plasmid are white and negative colonies (without plasmid or with empty plasmid) are blue.

8. Colony screening: For each eutopic cell culture, 10 positive white colonies were picked and screened to confirm positive insert before sending samples for Sanger sequencing to an external corporation (Eurofins Genomics). To this end, isolated white colonies were picked up with a tip on a pipette and material was washed away into a 1.5 mL tube containing 10 µL of ddH_2O. With the same tip, streaks were made on a Amp./2% X-gal agar plates and incubated O/N at 37 ºC. Tubes containing colonies were heated for 10 min at 95ºC and spin-down for 1 min at 13,200 rpm. A PCR reaction mixture was then prepared with 1.5 µL of supernatant, 10.2 µL of nuclease-free H_2O, 1.5 µL of 10x Taq buffer, 200 µM of dNTPs, 0.3 µL of Taq polymerase (Genaxxon bioscience), and 0.4 µM of both TOPO_forward and TOPO_reverse primers (Life Technologies) (**Table 4**) (Vf =15 µL). Then, a PCR program was run as follows: 1 cycle of 3 min at 95 ºC, 35 cycles of 30 s at 94 ºC, 30 s at 55 ºC and 1 min at 72 ºC. Additionally, a final step of 5 min at 72 ºC was performed before cooling-down and holding the reaction at 8 ºC.

Table 4: Primers used for colony screening (Chapter 5).

TOPO _Forward:	5'-ggaaacagctatgaccatgattacg -3'
TOPO _Reverse:	5'-gtaaaacgacggccagtgaattg -3'

Afterwards, colony PCR products were loaded into a 1% agarose (Biozym)-TPE-Gel red (1:25,000 dilution; Phoenix research) gel. Electrophoresis conditions: 80V, 60 min. 6 out of a maximum of 10 confirmed positive colonies (**Fig. 15**) were picked from the O/N incubated plate and seeded into an appropriate device for Sanger sequencing (Eurofins Genomics).

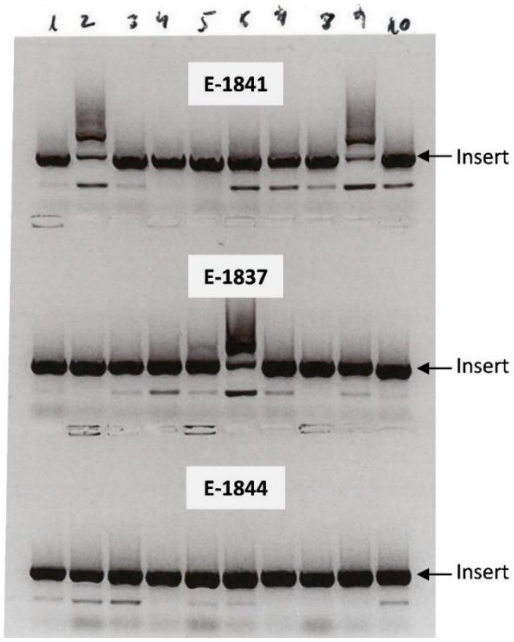


Figure 15. Electrophoresis of PCR product from white positive colonies. E-1841, E-1837 and E-1844 refers to the ID of the eutopic endometrial stromal cells from which genomic DNA was isolated. Insert refers to the 202 bp *VEGFA* fragment cloned into *E. coli* bacteria.

8. *In vitro* experiments

8.1. Peritoneal fluid stimulation of primary stromal cell cultures

To stimulate primary stromal cell cultures with PF pools (Chapter 2 and Chapter 3), cells were maintained for 4 h in FBS-free medium and subsequently treated and incubated for an additional 4 h with medium without PF (ØPF) or EPF or CPF at 25% in phenol red free DMEM-F12 (Thermofisher Scientific), following the protocol previously established by our laboratory (Cosín et al, 2010) (**Fig. 16**).

Whereas supernatants of the cell cultures were then collected, aliquoted on ice and frozen at -80 °C until protein quantification, adhered cells were washed with PBS at 4°C and frozen at -80 °C until total RNA extraction. All experiments were performed in triplicate.

Finally, to evaluate the influence of PF mixtures on the expression of the studied parameters, we calculated the difference between the levels obtained after treatment with and without PF in the cell cultures of the same patients. Subsequently, we correlated the results of miRNA levels with those of angiogenic and proteolytic factors to determine the relationship between them.

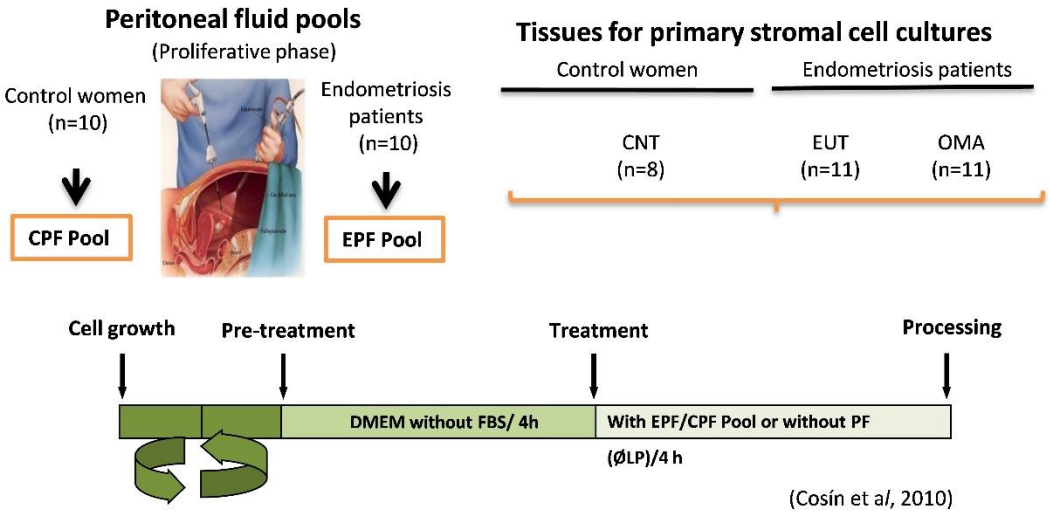


Figure 16. Schematic representation of study design for stimulation of primary stromal cell cultures with PF pools (Chapter 2 and Chapter 3). DMEM: Dulbecco's Modified Eagle's Medium. ØPF: without peritoneal fluid; CPF: control peritoneal fluid; EPF: endometriotic peritoneal fluid; CNT: control endometrium; EUT: eutopic endometrium; OMA: ovarian endometrioma.

8.2. Cell transfections

8.2.1. Cell transfections with miRNA mimics

To perform the miRNA mimic transfection (Chapter 3), cells were seeded 24h before transfections in DMEM (Thermofisher Scientific) in 10% FBS (Invitrogen) without antibiotics and transfected with chemically modified double stranded RNAs that either mimic endogenous miRNAs miR-16-5p, miR-29c-3p, miR-424-5p or are scramble RNA control. This was performed by using the siPORT^{TM} NeoFX^{TM} transfection agent (Life Technologies) in Opti®-MEM (Thermofisher Scientific) according to the manufacturer's instructions. Initially, dose-response experiments were performed in control cells with increasing doses of miRNA mimics (20 nM, 50 nM and 100 nM). Thus, we established 100 nM as the optimal dose for subsequent experiments. After 24 h, cells were collected for subsequent mRNA and protein analyses. All transfections were performed in triplicate.

8.2.2. Luciferase experiments

- *Plasmid construction*

To confirm the repressive action of miR-29c-3p through forming miRNA:*VEGFA* mRNA duplexes (Chapter 3), we inserted a fragment of the *VEGFA* 3′-UTR containing the binding site for this miRNA into a luciferase expression vector, generating the luciferase reporter construct pMIR *VEGFA*-3′UTR (**Table 5A**). Briefly, pMIR-*VEGFA*-3′UTR contained a fragment located at nt+1575-1829 of the *VEGFA* 3′UTR. The PCR fragment was cloned into the pCR 2.1 vector (Life Technologies). Positive clones were digested with SacI and HindIII (New England Biolabs) and the insert was subcloned into the luciferase reporter plasmid pMIR-REPORT^{TM} (Life Technologies) previously digested with SacI and HindIII (**Table 5B**). Insertion of the *VEGFA* 3′-UTR fragment was checked by sequencing into an ABI3130 XL instrument (Life Technologies). All sequence analyses and alignments were performed with the SeqmanPro program, LaserGene version 7.1 (DNASTAR).

To generate mutations in the predicted target site for the miR-29c-3p, seven nucleotides located in the seed sequence were deleted using the Quik-Change site-directed mutagenesis kit (Agilent Technologies) (**Table 5A**). Sequencing was performed with specific primers (**Table 5C**) to check for the deletion of the seed sequences into an ABI3130 XL instrument (Life Technologies). The primers used for cloning and mutagenesis are detailed in **Annex 2; Suppl. Table 2**.

- *Luciferase vector transfection*

miR-29c-3p mimic was co-transfected with pMIR-*VEGFA*-3′-UTR and Renilla vector pRL-TK (Promega) into the HCT-DK cell line (**Fig. 17)**. Cells were seeded at a density of 80,000 cells/well in 24-well plates with McCoy's 5A supplemented with 10% fetal calf serum (Sigma Aldirch) without antibiotics. After 24 h, cells were co-transfected with scrambled precursor (SCR) or miR-29c-3p mimic (both pMIR-REPORT plasmids—1000 ng/well—wild type or mutated for the miRNA seed site; Promega) and 100 ng/well of Renilla luciferase control plasmid (pRL-TK, Promega) using Lipofectamine LTX (Life Technologies), according to the manufacturer's instructions. Luciferase assays were

performed as previously described (Salloum-Asfar et al, 2014). The enzymatic activities of Renilla and firefly luciferases were quantified in a Synergy 2 luminometer (Biotek). Each combination of pMIR-REPORT (wild-type and mutated 3′UTR) and pRL-TK was tested in triplicate in five independent experiments. Firefly luciferase activity was normalized to Renilla luciferase activity for each transfected well. The normalized data were expressed as changes relative to the data of the cells transfected with 100 nM SCR mimic. SCR was taken as 100%.

Table 5: Primers used to clone the *VEGF-A* 3'UTR wild type (WT) and mutated (Mut) fragments (**Annex 1, Paper 3**) .

A: Cloned *VEGF-A* 3'UTR fragment. Highlighted (bold and underlined) are nucleotides deleted in the mutated fragment.

TTTGGCAACTTGTATTTGTGTGtatatatatatatatgtttatgtatatatgtgattctgataaaatagacattgctattctgtttt
ttatatgtaaaaacaaaacaagaaaaaatagagaattctacatactaaatctctctccttttttaattttaatatttgttatcatttatttat**tggtgct**act
gtttatccgtaataattgtggggaaaagatattaacaTCACGTCTTTGTCTCTAGTGCAG

B: Primers employed to clone the WT fragment.

VEGFA-sacI-F5: gagctctttggcaacttgtatttgtgtg
VEGFA-HindIII-R5: aagcttctgcactagagacaaagacgtga

C: Primers employed to clone the Mut fragment.

Mutated VEGFA_del29c_Forward:
atctctctccttttttaattttaatatttgttatcatttatttatttactgtttatccgt aataattgtg

Mutated VEGFA_del29c_Reverse:
cacaattattacggataaacagtaaataaataaatgataacaaatattaaaattaaaaaaggagagagat

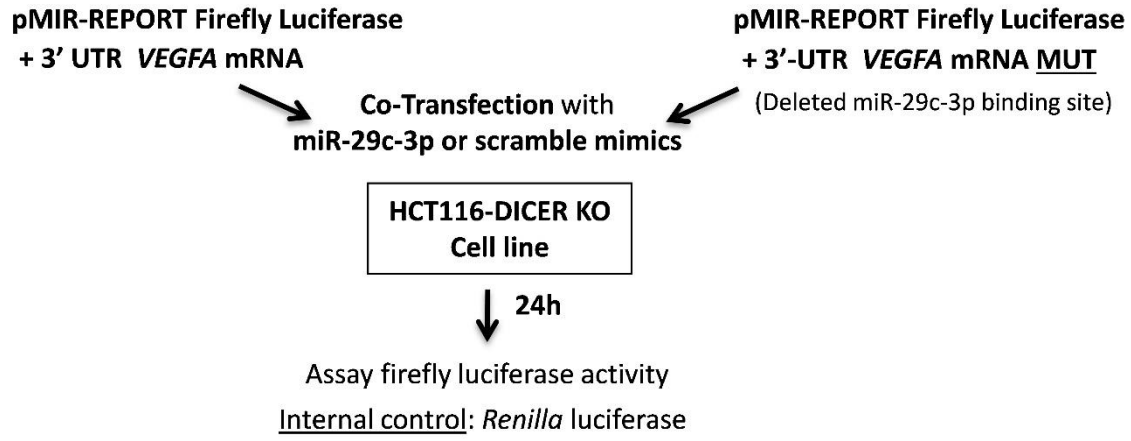


Figure 17. Schematic work-flow for miR-29c-3p:*VEGFA* mRNA luciferase experiments (Chapter 3). UTR: Untranslated region.

8.2.3. Cell transfections for targeted DNA methylation

- *Epigenetic editing with ZF proteins*

1. *Transfections with FuGENE® HD reagent (Promega):* $2 \cdot 10^5$ primary eutopic stromal cells/well (6-well plate format) and incubated with DMEM/10% FBS until they reached 80% of confluency. Culture media was changed prior to transfection. Transfection with FuGENE® HD reagent (Promega) were performed following the recommendations of the supplier. In each experiment, a total amount of 2.3 µg of VAZF3a3L plasmid (Dnmt3a-C, Dnmt3L and specific ZFP) and 6.9 µL of the transfection reagent were mixed with 900 µL of DMEM and incubated at RT for 20 min before adding the mixture to the cells. No change media was performed. Confocal microscopy analyses were scheduled every 24 h, up to a final time of 72 h. Then, cells were harvested by trypsinization and analysed by flow cytometry. Experiments were performed in triplicate.

   As a positive control, $1 \cdot 10^6$ HEK-293 cells were seeded in one well (6-well plate format) and transfected at a confluency of 80%. Culture media was change prior to transfection. Transfection with FuGENE® HD reagent (Promega) were performed following the recommendations of the supplier. In each experiment, a total amount of 1.5 µg of VAZF3a3L plasmid (Dnmt3a-C, Dnmt3L and specific ZFP) and 5 µL of the transfection reagent were mixed with 145 µL of DMEM and incubated at RT for 20 min before adding the mixture to the cells. No change media was performed. Confocal microscopy analyses were scheduled every 24 h, up to a final time of 72 h. Then, cells were harvested by trypsinization and analysed by flow cytometry. Experiments were performed in triplicate.

2. *Transfections with Lipofectamine™ 3000 reagent (Life Technologies):* primary eutopic stromal cells were seeded at a density of $2 \cdot 10^5$ cells/well (6-well plate format) and transfections were performed after 24 h, when cells reached 80% of density. Cells were seeded at a density of $2 \cdot 10^5$ cells/well in a 6-well plate and transfections were performed after 24 h, when cells reached 80% density. Either increasing volumes (3.5 µL, 7.5µL, reagent titration) or fixed volumes (3.75 µL, optimized dose) of Lipofectamine® 3000 reagent (Life Technologies) were diluted in 125 µL of Opti®-MEM medium (Life Technologies). 2.5 µg of total DNA (VAZF3a3L plasmid + GFP plasmid + LNGFR plasmid) were diluted in 125 µL of Opti®-MEM medium (Life Technologies) and 5 µL of P3000™ reagent (Life Technologies). Diluted Lipofectamine® 3000 reagent and DNA were mixed and incubated for 5 min at RT. Then, 250 µL of the mixture was added to the wells. 4 h post-transfection, culture medium was discarded, wells were washed with 1 mL of PBS and incubated with 2 mL of complete medium at 37 ºC, 5% CO_2. Analysis were performed in triplicate 24 h post-transfection, when cells were trypsinized and harvested.

3. *Transfections with Lipofectamine™ LTX reagent (Life Technologies):* primary eutopic stromal cells were seeded at a density of $2 \cdot 10^5$ cells/well (6-well plate format) and transfections were performed after 24 h, when cells reached 80% of density. Either increasing volumes (5 µL, 7.5 µL, 10 µL, reagent titration) or fixed volumes (7.5 µL, optimized dose) of Lipofectamine™ LTX reagent (Life

Technologies) were diluted in 125 µL of Opti®-MEM medium (Life Technologies). 2.5 µg of total DNA (either VAZF3a3L plasmid or pAdtrack empty vector (Addgene) + GFP plasmid + LNGFR plasmid) were diluted in 125µL of Opti®-MEM medium (Life Technologies) and 2.5 µL of PLUS™ reagent (Life Technologies). Diluted Lipofectamine® 3000 reagent and DNA were mixed and incubated for 5 min at RT. Then, 250 µL of the mixture was added to the wells. 4 h post-transfection and then every 48 h, culture medium was discarded, wells were washed with 1 mL of PBS and re-incubated with 2 mL of complete medium at 37 ºC, 5% CO_2. Analysis were performed in triplicate either 24 h (titration) or every 24 h up to 120 h post-transfection. The duration of 120 h post-transfection for maximum targeted DNA methylation delivery was optimized in previous papers of the laboratory where the PhD student performed his stay (Kungulovski et al, 2015) (**Fig. 18**).

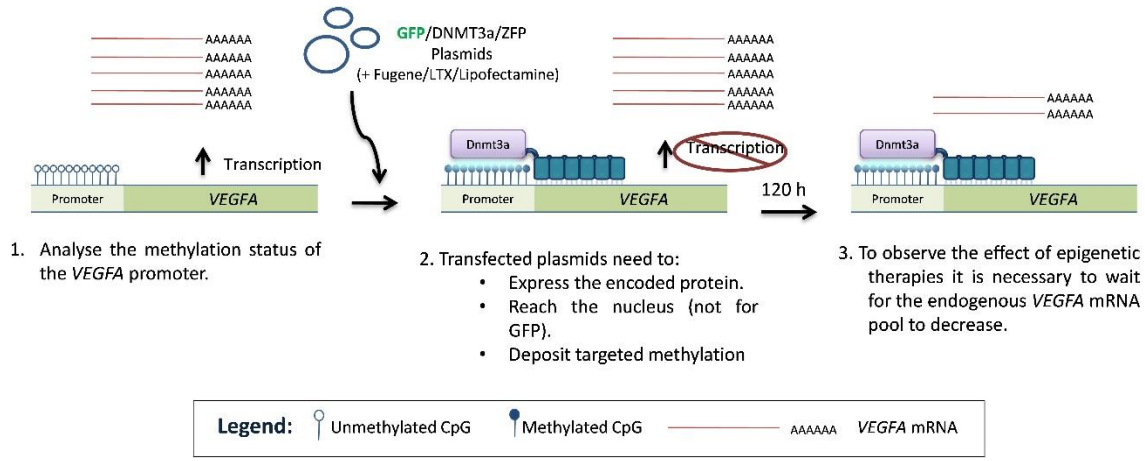


Figure 18. Schematic model of targeted DNA methylation at *VEGFA* promoter (Chapter 5).

8.3. MACSelect for transfected cell enrichment

Primary eutopic stromal cells transfected with Lipofectamine™ 3000 reagent were enriched 2 days after transfection using MACSelect™ LNGFR System (Miltenyi Biotec) following manufacturer's recommendations.

8.4. Analyses of transfection efficiency with flow cytometry

The percentage of the GFP positive cells was quantified using FACS analysis (BD FACS Calibur) with an acquisition setting of 10,000 events. For MACSelect enriched cells, analyses were performed in transfected but unlabelled cells, cells unretained in the magnetic field (flow-through) and enriched cells.

9. Statistical analyses

Statistical analyses were performed using the statistical package SPSS v.20 for Windows, considering statistically significant a p-value <0.05. The number of samples was calculated using the independent group comparison formula, with bilateral contrast analysis and Type I and II errors fixed at 5% and 20%, respectively. The Kolmogorov-Smirnov-Lilliefors normality test was applied to the continuous quantitative variables. To compare the means of these continuous quantitative variables with normal distribution, Student's t-test or ANOVA test was used, as appropriate by the number of groups analysed. If non-standard criteria were met, non-parametric tests were applied. For the study of correlations between two variables, the Pearson correlation method was used.

Nevertheless, "-omic" data requires a special statistical treatment, described as detailed:

9.1. Microarray data analysis

For miRNA expression microarray data analysis (Chapter 2 and Chapter 3), Affymetrix ".cel" data files were imported into Genomic Suite software (PARTEK) and normalized using the Robust Multi-array Analysis (RMA) algorithm. Following the miRNA expression workflow, normalization of data included RMA, background correction, quartile normalization, log2 transformation values and median polish according to the Genisphere indications for the FlashTag Biotin labelling kit. After ANOVA statistical analysis, the miRNA generated lists were used for further analysis including only miRNAs with a p-value ≤ 0.05.

Principal component analysis (PCA) was performed in all array data from hybridized samples, so they were reviewed according to their characteristic miRNA expression profiles. Hierarchical clustering representation of differentially expressed miRNA from all studied samples allowed to identify samples with similar of miRNA expression patterns, according to p-values and fold-change criteria. Compared with PCA supervised hierarchical clustering represents, only the generated lists of miRNAs that are differentially expressed.

9.2. DNA methylation profiling data analysis

Following common assumptions (Krausz et al, 2012; Houshdaran et al, 2016), CpGs were defined as hypermethylated if their β-values ≥ 0.8; hypomethylated if β-values ≤ 0.2; and intermediately methylated if $0.2 \leq$ β-values ≤ 0.8. For all comparison, only $\Delta\beta \geq 0.136$ were considered, since differences under this cipher could be due to background noise and platform variability (Bibikova et al, 2009).

In order to detect the CpGs that could separate between groups, a regression model using elastic net was employed, capable of dealing with databases with many more variables than observations in a consistent way and with the ability to select the important variables penalizing the rest to zero (Zou et al, 2005). For the selection of the penalty factor, 100 repetitions of cross validation of 3 iterations were used. The lambda value was chosen following the rule of a standard error (Friedman et al, 2001).

For the analysis of conserved CpGs among individuals, we calculated standard deviation (SD) of mean β-values. CpGs with SD <0.2 were considered as "conserved", following established criteria (Krausz et al, 2012).

DMRs were identified using 'seqlm' package (https://github.com/raivokolde/seqlm) in the R environment, utilising MDL-based approach. The Benjamini–Hochberg FDR was calculated for each probe, with an FDR corrected p-value <0.05 used to define DMRs.

These analyses were performed at the Unit of Data Science, Biostatics and Bioinformatics of our institution.

9.3. Analysis of bisulphite sequencing results

Sanger sequencing data of bisulphite treated samples was provided by an external corporation (Eurofin genomics). Afterwards, data was analysed employing established protocols (http://services.ibc.uni-stuttgart.de/BDPC/BISMA/manual_unique.php).

IV. Results

IV. Results

Chapter 1. microRNA expression profiles in different endometrial tissues from control women and endometrial and endometriotic tissues from patients with endometriosis.

Chapter 2. Validation of an *in vitro* model of endometriosis to study the pro-angiogenic and pro-fibrinolytic properties of peritoneal fluid from patients with endometriosis.

Chapter 3. Effect of peritoneal fluid on the miRNA expression profiles of endometrial and endometriotic cells from women with endometriosis.

Chapter 4. Epigenomic landscape in endometriosis. Analysis of the genome wide DNA methylation.

Chapter 5. Epigenetic editing of the *VEGFA* promoter in primary endometrial stromal cell cultures.

Chapter 1

Chapter 1. microRNA expression profiles in different endometrial tissues from control and endometrial and endometriotic tissues from patients with endometriosis

In vitro studies represent an essential and common laboratory tool for understanding cell behaviour under controlled experimental conditions. However, there are several limitations inherent to its experimental design, given the tissue complexity in terms of cellular composition, ECM, vascularization, as well as possible autocrine, paracrine and hormonal signalling. In order to gain an overview of miRNA-mediated regulation in endometriosis, we wished to study miRNA profiles in different ectopic lesions as well as in endometria from both patients and control women.

1.1. Clinicalcharacteristics of patients included in the study

Characteristics of patient of study are provided in **Table 6**.

To achieve the objectives proposed in this chapter, we studied 51 paired EUT and OMA samples, 18 PIs and 20 RVNs obtained at surgery from 51 Caucasian women with endometriosis (mean age: 34.0 years, range: 20–45).

CNT tissues were obtained from 32 Caucasian asymptomatic women with confirmed absence of endometriosis (mean age: 36.4 years, range: 27–45) who underwent surgery for laparoscopic tubal sterilization.

At the time of surgery and specimen collection, 26 (51%) women with endometriosis were in the proliferative phase and 25 (49%) were in the secretory phase of the menstrual cycle. Regarding control women, 15 (47%) were in the proliferative phase and 17 (53%) were in the secretory phase of the menstrual cycle. In this study, we excluded women in the menstrual phase.

Table 6: Clinical characteristics of patients included in the study.

	CONTROL WOMEN (n = 32)	**ENDOMETRIOSIS** (n = 51)
AGE (YEARS; MEAN± SEM) **RANGE** (YEARS)	36.4 [27 - 45]	34.0 [20 - 45]
CYCLE PHASE		
• PROLIFERATIVE	n = 15 (47%)	n = 26 (51%)
• SECRETORY	n = 17 (53%)	n = 25 (49%)
• MENSTRUAL	n = 0 (0%)	n = 0 (0%)
ENDOMETRIOTIC LESIONS		
• OMA	NA	n = 51 (100%)
• PI	NA	n = 18 (35.3%)
• RVN	NA	n = 20 (39.2%)
STAGE		
• I-II	NA	n = 0 (0%)
• III-IV	NA	n = 51 (100%)

NA: not applicable

1.2. miRNAs expression profiles in endometrial and endometriotic tissues determined by microarray analysis

miRNAs expression profiles in endometrial tissues and endometriosis determined by microarray analysis expression profiles of miRNAs were determined on the Gene Chip microarray miRNA 2.0 Array (Affymetrix Platform, Array Service, IIS La Fe), which includes probes for 1,105 mature miRNAs and 1,105 pre-miRNAs. Expression analyses were determined on 7 samples of EUT, 3 samples of OMA and 5 tissue samples of CNT from women without endometriosis.

The results obtained in the microarray were analysed using the software Partek Genomic Suite. PCA showed that whereas EUT tissues had expression profiles relatively close to those of CNT tissues, the expression profiles in OMAs were clearly different from those coming from endometria, regardless of the presence of endometriosis. Statistical analysis identified 157 miRNAs distinctly expressed (±1.3-FC; p <0.05; 79 up-regulated and 78 down-regulated) in OMA and/or in EUT samples compared to CNT tissues (**Annex 2, Suppl. Table5**).

Further clustering analysis of miRNAs distinctly expressed by supervised hierarchical cluster showed relatively close patterns of miRNA expression between EUT and CNT, while samples of OMA separately grouped (**Fig. 19A and 19B**).

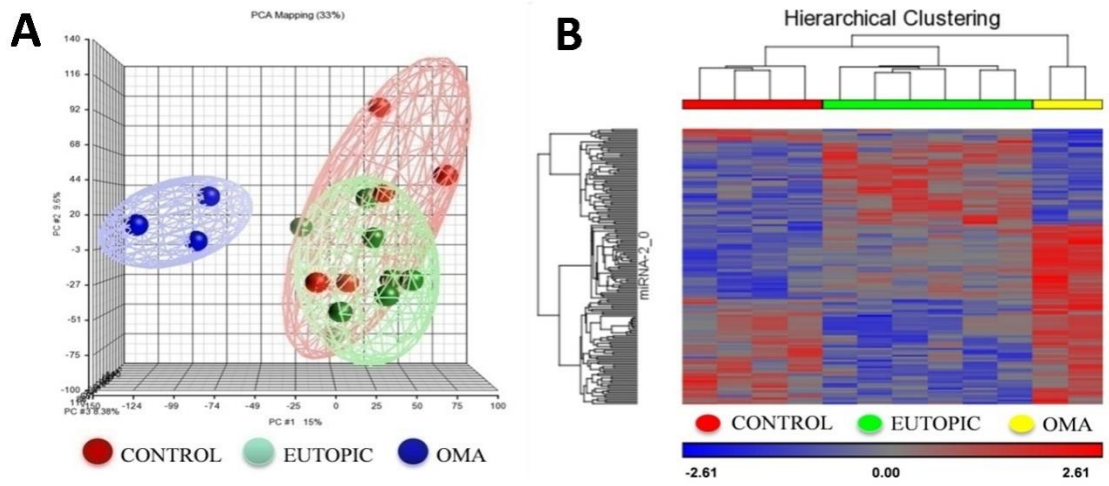


Figure 19. Graphical algorithms showing the expression profiles of miRNAs in different tissues. A) Principal Component Analysis (PCA) applied to the expression of all probes in the Affymetrix GeneChip miRNA 2.0 array. B) Hierarchical cluster analysis of miRNAs differently expressed from different tissues. Control: control endometria; Eutopic: eutopic endometria; OMA: ovarian endometrioma (**Annex 1, Paper 1**).

We next performed an *in silico* study to determine the genes predicted to be targeted by the 157 differentially expressed miRNAs (±1.3-FC; p <0.05) and investigate which of them might regulate the expression of the most important factors involved in angiogenesis and fibrinolysis, or that had been implicated in endometriosis. This enabled us to select 12 miRNAs for validation by qRT-PCR in a larger cohort of samples (**Table 7**). 6 out of the 12 miRNAs were up-regulated (namely miRNAs miR-29c-3p, -138-5p, -202-3p, -411-5p,

-411-3p, -424-5p) in OMA and/or EUT *vs*.CNT and 6 miRNAs were down-regulated (namely miRNA miR-16-5p, - 373-3p, -449b-3p, -556-3p, -636, -935).

It is important to clarify that Gene Chip microarray miRNA 2.0 Array (Affymetrix) identifies miRNAs according to miRBase version 16. Nevertheless, some miRNAs have been renamed in updated versions of this database. Thus, we intended to avoid ambiguities by providing current and former nomenclatures, together with the unique sequence that unequivocally identifies every miRNA (**Table 7**).

Table 7. miRNA microarray expression and targets of miRNA selected for the qRT-PCR experiments (**Annex 1, Paper 1**)

			EUT *vs* CNT		**OMA *vs* CNT**		
miRNA (v. 16)[a]	**miRNA (v. 20)[b]**	**miRNA sequence (5´→3´)**	**FC**	***p***	**FC**	***p***	
miR-16	miR-16-5p	U**AGCAGCAC**GUAAAUAUUGGCG	-1.0680	0.00073	1.00307	0.772305	VEGFA, EGFR2, BCL2, FGFR1, COX2
miR-29c	miR-29c-3p	U**AGCACCAU**UUGAAAUCGGUUA	1.5202	0.00588	1.19676	0.0998617	VEGFA, PDGFB-C, THSD4 (TSP-1D4), SERBP1, ADAMTS2, 5-7, 9, 17-19
miR-138	miR-138-5p	A**GCUGGUGU**UGUGAAUCAGGCCG	1.1497	0.00012	1.49604	0.0285628	ADAMTS5, BCL2, TNFF4
miR-202	miR-202-3p	A**GAGGUAUA**GGGCAUGGGAA	6.1602	0.00338	1.11787	0.496386	THBS1 (TSP-1), GLI1[c], IL6R, MMP1, FGF5, FGF11, IL6, IL10
miR-373-star	miR-373-3p	G**AAGUGCU**UCGAUUUUGGGGUGU	-1.5987	0.01366	-1.5995	0.0108726	VEGFA, IL8, ADAMST18, MMP24, TIMP3,ESR1
miR-411	miR-411-5p	U**AGUAGAC**CGUAUAGCGUACG	1.3027	0.01779	1.0831	0.208284	CDH2, ADAMST19
miR-411-star	miR-411-3p	U**AUGUAAC**ACGGUCCACUAACC	2.4150	0.01738	1.73618	0.0198753	ADAMTS1, HIF1α, CDH2
miR-424	miR-424-5p	C**AGCAGCA**AUUCAUGUUUUGAA	1.1331	0.01904	1.22434	0.178638	VEGFA, IL1, FGF2
miR-449b*	miR-449b-3p	C**AGCCACA**ACUACCCUGCCACU	-2.1091	0.02845	-1.09801	0.408264	MMP-16, IL6R, PDGFRA, PDGFRB
miR-556-3p	miR-556-3p	A**UAUUACC**AUUAGCUCAUCUUU	-1.3774	0.06692	-1.42387	0.0011172	VEGFA, ADAMST1, SERBP1, CDN7
miR-636	miR-636	U**GUGCUUG**CUCGUCCCGCCCGCA	-1.7670	0.12815	-1.52172	0.0488759	ADAMTS14, SERBP1, PDGFRA, FGF12
miR-935	miR-935	C**CAGUUAC**CGCUUCCGCUACCGC	-1.6869	0.84800	-1.4834	0.0154318	SERBP1, FGF1

[a] Referred to miRBase database release version 15.
[b]Referred to miRBase database release version 20.
[c](Zhao et al, 2013).

1.3. Quantification of selected miRNAs by qRT-PCR in endometrial tissues and endometriotic lesions

Validation by qRT-PCR of the expression levels of the 12 selected miRNAs was performed in a cohort of 32 CNT samples and 51 paired samples of EUT and OMA. In addition, these miRNAs were also quantified in 18 PI and 20 RVN obtained from the same 51 patients. This served to determine if these miRNAs were also deregulated in ectopic lesions.

1.3.1. miRNAs in tissues of patients compared to the control endometrium

The results of the validation phase showed that the EUT expresses lower levels of miRNAs miR-202-3p, -424-5p, -449b-3p and -556-3p and that OMA expresses higher levels of miR-29c-3p, -138-5p, -202-3p, -373-3p and -411-5p in relation to the CNT. OMA and RVN lesions presented lower levels of miR-449b-3p compared to CNT. Notably, the expression of miR-202-3p in OMA is hundred times greater than that in CNT. In PI and RVN, an increased expression of the miRNAs miR-29c-3p, -138-5p, -202-3p, -16-5p, -411-5p, and -424-5p *vs.* both EUT and CNT was observed (**Fig. 20**).

1.3.2. miRNAs in the different endometriotic lesions incomparison to the eutopic endometrium

The levels of the miRNAs miR-29c-3p, -138-5p, -411-5p, -424-5p in endometriotic lesion samples (OMA, PI and RVN) were higher than those observed in EUT of patients (*p* <0.001). In addition, over-expression of miR-202-3p in OMA *vs.* EUT (*p* <0.001) was approximately two hundred times (**Fig. 20**).

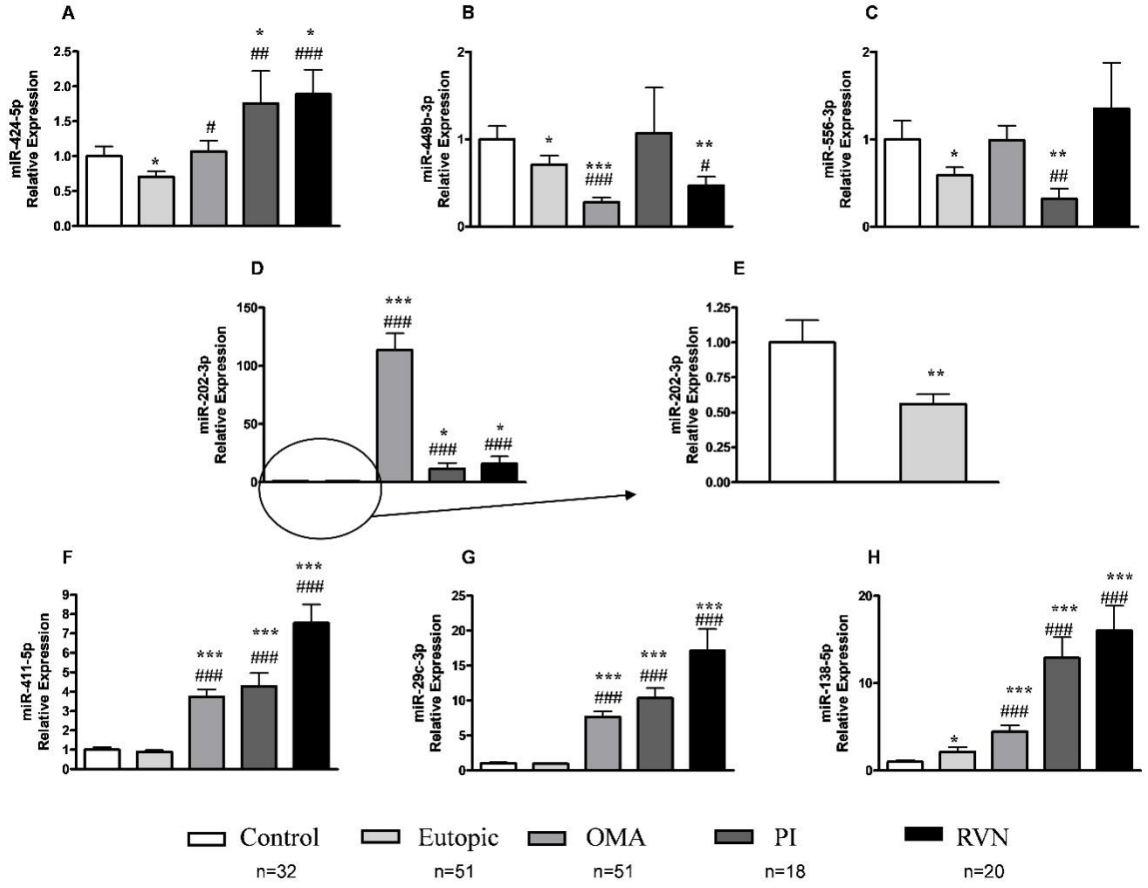


Figure 20.Validation of selected tissue miRNAs by qRT-PCR. A) miR-424-5p; B) miR-16-5p; C) miR-556-3p; D) and E) miR-202-3p; F) miR-29c-3p; G) miR-373-3p. Relative expression *vs.* control (endometrium control=1). Mean ± SEM. *p <0.05, **p <0.01, ***p <0.001 *vs.* control. #*p* <0.05, ##*p* <0.01, ###*p* <0.001 *vs.* eutopic endometria. Control: control endometria; Eutopic: eutopic endometria; OMA: ovarian endometrioma; PI: peritoneal implant; RVN: nodule in the rectovaginal septum (**Annex 1, Paper 1**).

1.4. Quantification of angiogenic and fibrinolytic components in different tissues

In our study, EUT showed statistically significant increased levels of VEGF-A in relation to the CNT without significant changes in its inhibitor, TSP-1. In contrast, statistically significant reduced levels of VEGF-A in OMA vs EUT (p <0.01) and PI (p <0.001) were observed. PI showed increased levels of VEGF-A, both in relation to the CNT and EUT, with no significant changes in TSP-1 levels. Finally, RVN showed significant increased values in the expression of TSP-1 (p <0.05) in relation to CNT (**Fig.21A-B**), being the largest increase observed in OMA.

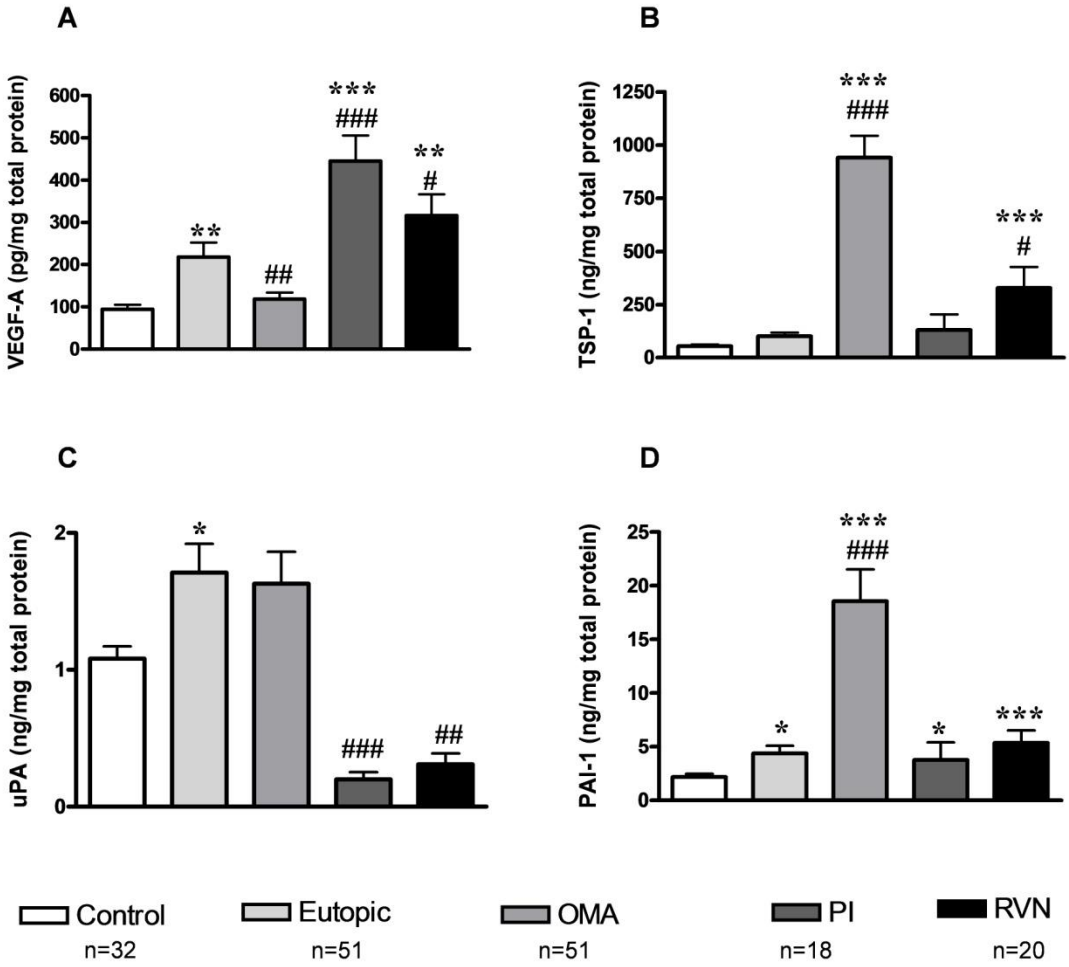


Figure 21. Protein levels of A) VEGF-A; B) TSP-1, C) uPA and D) PAI-1 in different tissues. Mean ± SEM. *p <0.05, **p <0.01, ***p <0.001 *vs.* control. #p <0.05, ##p <0.01, ###p <0.001 *vs*. eutopic. Control: control endometria; eutopic: eutopic endometria; OMA: ovarian endometrioma; PI: peritoneal Implant; RVN: rectovaginal nodule (**Annex 1, Paper 1**).

1.5. Correlation between distinctly expressed miRNAs and angiogenic factors in tissue extracts

The relationship between the expression of the miRNAs and their target proteins is inverse as a consequence of its mechanism of action. Therefore, a significant inverse correlation between the levels of miR-424-5p and VEGF-A both in EUT (r= -0.380, *p*= 0.006) and CNT (r= -0.352, $p < 0.05$) endometrium was observed (**Fig. 22**). We also observed an inverse and significant correlation between levels of miR-449b-3p and TSP-1 (r = -0.314, *p*= 0.02) in OMA.

1.6. Discussion

The expression profiles of miRNAs in EUT as well as in ectopic lesions of women with endometriosis have recently been described (Burney et al, 2009; Ohlsson-Teague et al, 2009; Filigheddu et al, 2010; Laudanski et al, 2013) but few of them have determined the differences in levels of expression between the endometrium of patients and controls (Burney et al, 2009; Laudanski et al, 2013).Two review articles summarizing all previous studies assessing miRNA expression in endometriosis have recently been published by our group (Marí-Alexandre et al, 2016a, 2016b).

In our study, the miRNAs expression profile analysed by array in endometrial and endometriotic tissues showed that 157 mature miRNAs were distinctly expressed in OMAs and/or EUT compared to CNT endometria. The *in silico* study allowed to select 12 miRNAs involved in the regulation of angiogenesis for its validation in a greater number of samples, as well as in other endometriotic lesions, including PIsand RVNs.

The results of qRT-PCR validation showed that the expression of miR-202-3p was significantly decreased in the EUT in relation to CNT. However, this miRNA was observed to be considerably overexpressed in the OMA, its levels being approximately hundred times those of the CNT. Recent studies (Zhao et al, 2013) describe that miR-202-3p decreases the expression of homologous oncogenic transcription factor associated with glioma I (GLI I), which in turn regulates the expression of genes involved in proliferation, migration, invasion, and angiogenesis through signaling pathways that involve BCL-2, CD24, metalloprotease 2 and 9 (MMP2 and MMP-9) proteins (Carpenter and Lo, 2012). GLI I also regulates the transcription of VEGF-A (Cao et al, 2012; Carpenter and Lo, 2012; Zhao et al, 2013). In addition, it has been reported that the anti-apoptotic protein BCL-2 is overexpressed in the EUT of women with endometriosis (Burney et al, 2009, Burney and Giudice, 2012). This overexpression of BCL-2 could be mediated by the miR-202-3p through the transcriptionfactor GLI I. Therefore, significantly reduced levels of miR-202-3p may contribute to elevated levels of VEGF-A observed in EUT. In contrast, the large increase in miR-202-3p expression observed in the OMA could explain, at least in part, the low angiogenic and invasive capacity of this tissue.

Regarding angiogenesis, our study showed that the expression of VEGF-A in endometriotic lesions showed a clear difference in relation to CNT. OMAs showed reduced levels of VEGF-A expression compared to EUT ($p < 0.01$) and to PI ($p < 0.001$). However, these ovarian lesions presented with the largest increase in the expression of the angiogenic inhibitor TSP-1. Taken together, these results denote the low angiogenic

capacity of this type of lesion, usually observed in the endometriotic lesions in advanced stages of the disease (Nisolle et al, 1993).

PIs showed increased levels of VEGF-A in relation to CNT, EUT and OMA, and lower levels of miR-556-3p than CNT and OMA. However, TSP-1 levels did not show significant changes in relation to endometrium from control women. Overall, these data are in line with the angiogenic activity of the primary red peritoneal lesion.

Finally, significantly increased levels of VEGF-A in the eutopic endometrium in relation to the control reflect the greater angiogenic capacity of this tissue, which could contribute to the survival of migrated to the peritoneum tissue during the menstrual phase. No significant changes in expression of TSP-1 were observed in relation to CNT.

miRNAs are able to act in conjunction with other miRNAs in the 3'-UTR of a particular mRNA through the *principle of coordinated action*, increasing the repressive effect on the translation of a gene. In our study, two of the validated miRNAs whose target is VEGF-A, miR-424-5p and miR-556-3p, showed a reduced expression in EUT relative to CNT. In addition, the levels of VEGF-A are increased in the EUT, being able to inversely and significantly correlate with the observed miR-424-5p levels. Remarkably, the binding of both miRNAs to the *VEGFA* 3'-UTR is not sterically hindered, since seed region of miR-556-3p binds at positions 1760-1766 and that of miR-424-5p binds at positions 276-283. Taken together, these observations suggest that the reduce expression of both miRNAs could explain, at least in part, the greater activity angiogenic activity observed in EUT.

As for PIs, reduced levels of miR-556-3p and increased levels of its target, VEGF-A, were observed in relation to OMA and CNT. Thus, this miRNA may also contribute to elevated levels of VEGF-A in this tissues. In this sense, the increase of angiogenesis in the endometrium would be necessary for the initial formation of active PIs and the development of a vascular network that facilitates the growth and invasion of the ectopic tissue.

The present study indicates that miR-29c-3p is more abundant in endometriotic tissues compared to the endometrium of both patients and controls. Since the targets of this miRNA comprise different ECM components, our results reinforce previous studies that suggest the potential role of the miRNAs in the process of remodelling that leads to the implantation of the endometrial tissue outside the uterus and to the formation of endometriotic lesions (Ohlsson-Teague et al, 2009; Filighedu et al, 2010; Hawkins et al, 2011).

In conclusion, our study suggests a relevant role of miRNAs at tissue level in the aetiopathology of endometriosis, acting at the level of both angiogenic systems as proteolytic. The fact that higher expression of VEGF-A and uPA in ectopic lesions occurs without significant changes in the levels of their mRNAs suggest a post-transcriptional regulatory action mediated by miRNAs. The study of endometrial and endometriotic tissues allowed to show that there is a characteristic different expression profile in the endometrial tissue of women with endometriosis and in OMA in relation to the CNT endometrium. Validation by qRT-PCR of the selected miRNAs and their correlation with the protein levels of the main components of both angiogenic and fibrinolytic systems suggest the implication of a miRNA-mediated regulation in the establishment and survival of endometriotic lesions.

Chapter 2

Chapter 2. Validation of an *in vitro* model of endometriosis to study the pro-angiogenic and pro-fibrinolytic properties of peritoneal fluid from patients with endometriosis

In a previous study, our research group established an *in vitro* model for the study of angiogenesis and fibrinolysis in endometriosis (Cosín et al, 2010). At that time, we observed that PF from patients with endometriosis, at a final 25% concentration in culture medium, induced and increase of both *VEGFA* and *PLAU* mRNA and protein in primary endometrial stromal cell cultures from patients.

In the current study, we wished to validate this *in vitro* model not only in primary stromal cell cultures from endometrial samples but also from OMAs and, additionally, to evaluate whether the miRNAs regulating the expression of angiogenic and fibrinolytic factors could also be influenced by PF. Noteworthy, the miRNAs evaluated (namely miR-16-5p, -17-5p, -20a, -21, -125a, -221 and -222) had been chosen as we previously observed them to be potential regulators of angiogenesis and fibrinolysis in endometrial and endometriotic tissues (Ramon et al, 2011).

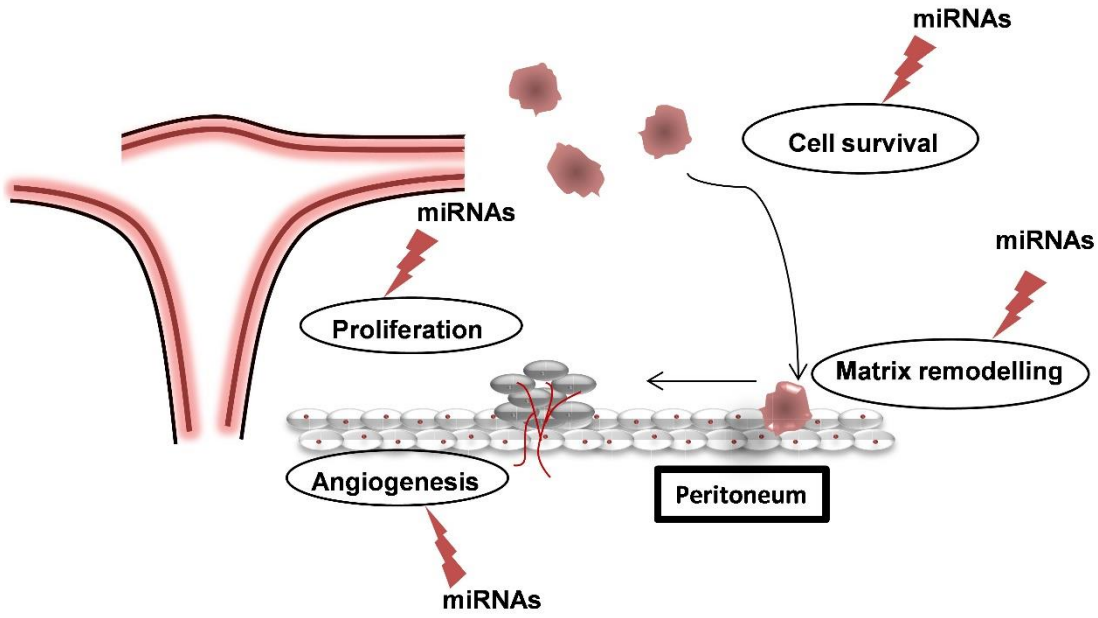


Figure 22. Schematic representation of peritoneal fluid influence on migrated endometrial fragments, potentially affecting mechanisms involved in the pathophysiology of the disease, as angiogenesis and matrix remodelling (**Annex 1, Paper 4**).

2.1. Characteristics of patients included in the study

Clinical characteristics of the original cohort are shown in **Table 8**.

Primary cell cultures were performed from samples of EUT and OMAs from 11 patients with moderate or severe endometriosis (stages III-IV) (mean age 32.4 years; range 19–40). Primary cell cultures of CNT were also performed from 8 samples of women without the disease (mean age 36.1 years; range 24–43). Importantly, no statistically significant differences were observed with respect to age between groups (EUT *vs.* CNT, p =0.312; OMA *vs.* CNT, p =0.116).

Moreover, pools from 10 PFs from women with endometriosis (endometriotic peritoneal fluid, EPF) (mean age: 33.1 years, range 27-39) and from 10 PFs from women without endometriosis (control peritoneal fluid, CPF) (mean age: 37.2 years, range 21-47) were performed. All participants were in the proliferative phase of their menstrual cycle at the time of sample collection.

These samples (primary cell cultures and PF pools) provided the study cohort for Chapter 2 and Chapter 3.

Table 8: Clinical characteristics of patients included in the study (Chapter 2 and Chapter 3).

Tissue samples for primary cell cultures	**CONTROL WOMEN** (n = 8)	**ENDOMETRIOSIS** (n = 11)
AGE (YEARS; MEAN± SEM)	36.1	32.4
RANGE (YEARS)	[24 - 43]	[19 - 40]
CYCLE PHASE		
• PROLIFERATIVE	n = 4 (50%)	n = 6 (54.5%)
• SECRETORY	n = 4 (50%)	n = 5 (45.5%)
• MENSTRUAL	n = 0 (0%)	n = 0 (0%)
STAGE		
• I-II	NA	n = 0 (0%)
• III-IV	NA	n = 11 (100%)

PF samples	**CONTROL WOMEN** (n = 10)	**ENDOMETRIOSIS** (n = 10)
AGE (YEARS; MEAN± SEM)	37.2	33.1
RANGE (YEARS)	[21-47]	[27 - 39]
CYCLE PHASE		
• PROLIFERATIVE	n = 10 (100%)	n = 10 (100%)
• SECRETORY	n = 0 (0%)	n = 0 (0%)
• MENSTRUAL	n = 0 (0%)	n = 0 (0%)
STAGE		
• I-II	NA	n = 0 (0%)
• III-IV	NA	n = 10 (100%)

NA: not applicable

2.2. Effect of peritoneal fluid on angiogenic factors in primary endometrial and ovarian endometrioma stromal cell cultures

In the absence of PF exposure (ØPF), a significant increase (p <0.05) was observed in the protein levels of VEGF-A in the OMA cell cultures (52.90 ± 16.45 pg/mL) and endometrial cell cultures of patients (37.14 ± 11.22 pg/mL) compared with endometrial cell cultures from control women (1.23 ± 0.07 pg/mL) (**Fig. 23**).

Both CPF and EPF significantly increased, in every cell type, the protein levels of VEGF-A compared to the corresponding cell culture without PF exposure (**Fig. 23**). Nevertheless, mRNA expression was not significantly modified by exposure to neither of the PF mixtures. The highest protein expression of VEGF-A was observed in cell cultures from ovarian endometrioma cells from women with endometriosis treated with EPF (**Fig. 23**). Regarding TSP-1, its expression was not significantly modified in any cell culture, regardless of the treatment with any of the PF pools (**Fig. 23**).

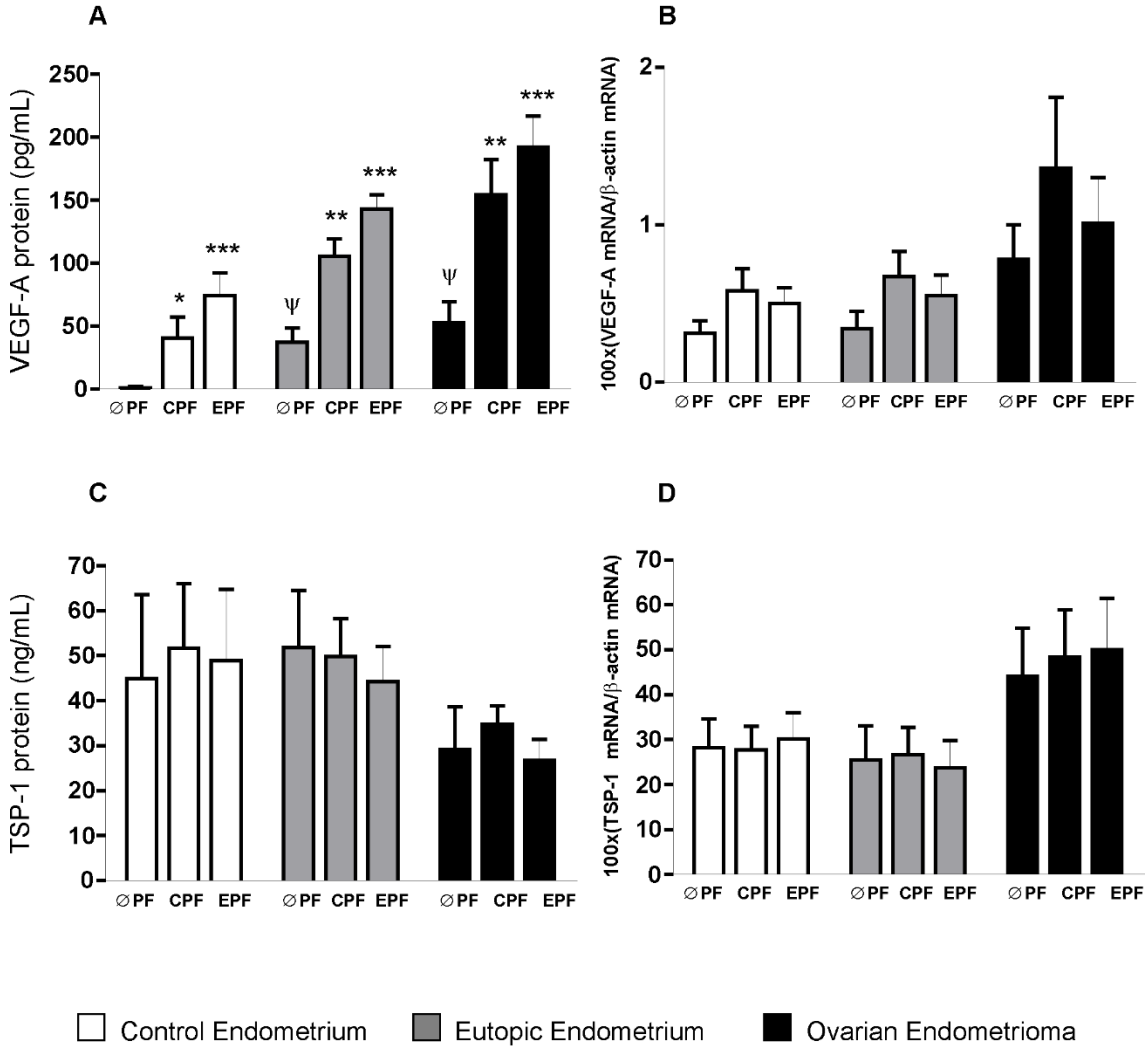


Figure 23. Effects of PF on the expression of VEGF-A and TSP-1 in stromal cell cultures from endometrial tissues of patients and control women and ovarian endometrioma tissue from patients. ØPF: without PF; CPF: control PF; EPF: endometriosis PF. Mean ± SEM. *p <0.05, **p <0.01, ***p <0.001 *vs.* ØPF from the same tissue culture. Ψ p <0.05 *vs.* ØPF from CNT endometrium. A) VEGF-A protein; B) *VEGFA* mRNA; C) TSP-1 protein; D) *THBS1* mRNA (**Annex 1, Paper 2**).

2.3. Effect of peritoneal fluid on fibrinolytic factors expression in cultures of stromal cells from different tissues

PF from both patients and control women induced a significant increase in the expression of both uPA (**Fig. 24A**) and PAI-1 (**Fig. 24C**) levels in all studied primary cell cultures without significantly modifying their mRNA levels (**Fig. 24B** and **24D**, respectively).

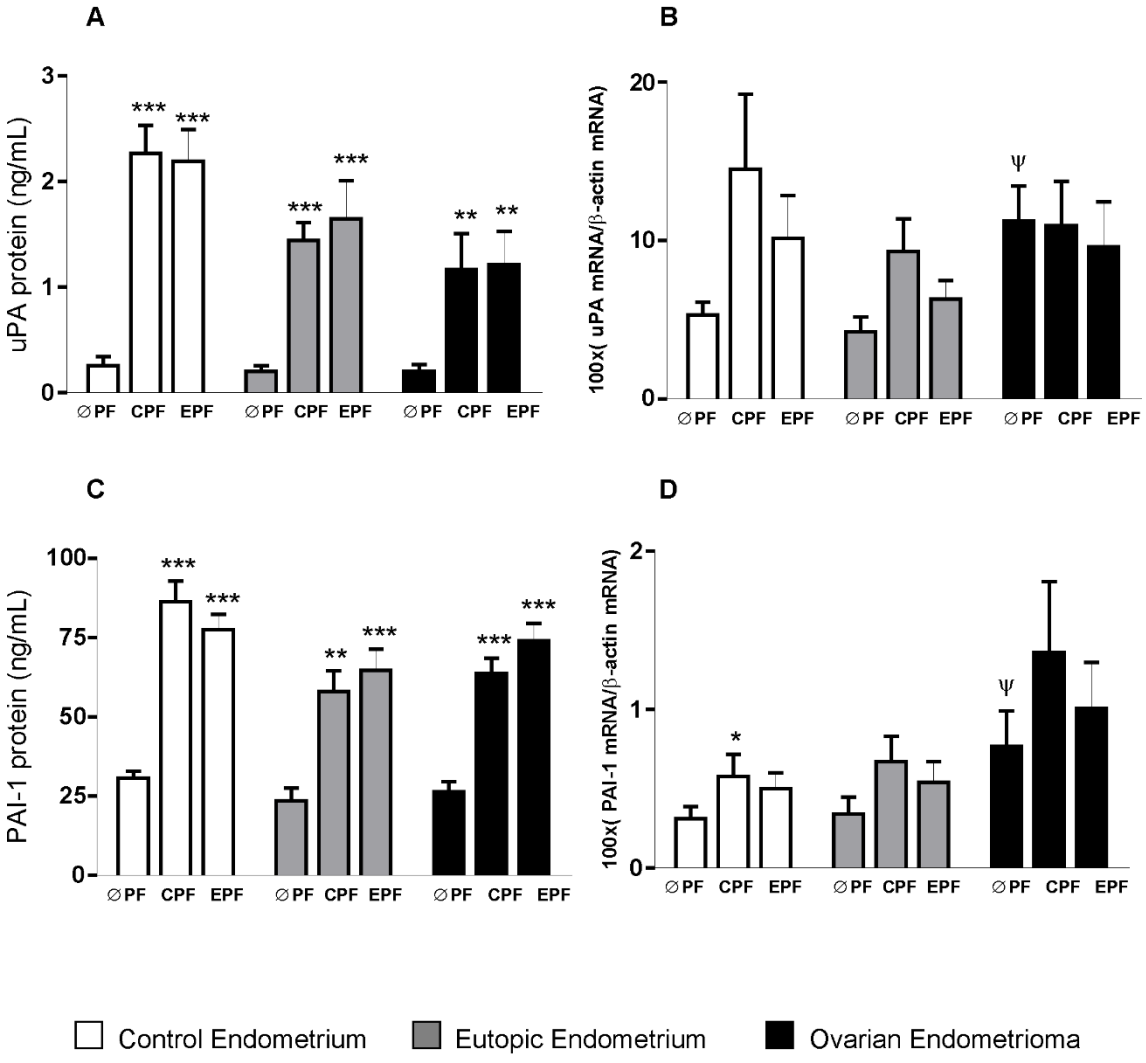


Figure 24. Effect of PF on the expression of uPA and PAI-1 in stromal cell cultures from endometrial tissues of patients and control women and from ovarian endometrioma tissues of patients. ØPF: without PF; CPF: control PF; EPF: endometriosis PF. Mean ± SEM. * p <0.05, ** p <0.01, *** p <0.001 *vs*. ØPF from the same tissue culture. Ψ p <0.05 *vs*. ØPF from control endometrium. A) uPA protein; B) *PLAU* mRNA; C) PAI-1 protein; D) *SERPINE1* mRNA (**Annex 1, Paper 2**).

2.4. Effect of peritoneal fluid on selected miRNA levels

In this validation phase, we considered 6 miRNAs (namely miR-16, -17-5p, -20a-5p, -125a-5p, -221 and -222) for qRT-PCR quantification, since we had previously evaluated their potential as regulators of the angiogenic and proteolytic factor involved in these experiments (Ramón et al, 2011).

The exposure of control endometrial cells (white bars) to both EPF and CPF significantly reduced the expression levels of the all the studied miRNAs. Moreover, both CPF and EPF also induced a reduction of all miRNA levels in cell cultures from endometrial tissue (grey bars) and ovarian endometriomas (black bars) of patients, though this reduction was not always statistically significant. Importantly, in all cases the greatest reduction was observed after exposure to EPF (**Fig. 25**).

2.5. Correlation between changes in miRNA expression and changes in angiogenic and fibrinolytic parameters after treatment with peritoneal fluid

In order to evaluate the influence of PF on the expression of the angiogenic and fibrinolytic factors, we assessed the difference between their levels in response to each treatment and the basal expression of each studied parameter. Thereafter, we correlated the differences observed in the expression of the angiogenic and fibrinolytic factors with the differences in the expression of miRNAs after treatment with each PF pools.

Hence, an inverse correlation between changes in the expression of VEGF-A and changes of miR-16 in OMA (r= -0.525, p= 0.018) (**Fig. 26A**) and endometrial (r= -0.733, p <0.001) (**Fig. 26B**) cell cultures from patients after treatment with PF pools was observed. In addition, a significant and inverse correlation between changes in VEGF-A protein levels and miR-17-5p (r= -0.739, p =0.001), miR-20a-5p (r= -0.676, p= 0.001), miR-125a-5p (r= 0.567, p= 0.01) and miR-222 (r= -0.494, p= 0.037) in OMA cell cultures after PF pools treatment was also observed.

2.6. Discussion

The aim of this study has been to validate an *in vitro* model in order to study the role of peritoneal microenvironment in endometriosis-related angiogenesis and fibrinolysis, by using primary stromal cell cultures from EUT and CNT samples and from OMA. Additionally, we aimed to test whether the miRNAs regulating the expression of angiogenic and fibrinolytic factors within the endometrial and endometriotic tissues (Chapter 1 and **Annex 1, Paper 1**) could also be influenced by PF.

Previous results (Cosín et al, 2010) indicated that PF is capable of interacting with endometrial cells and that EPF enhances the expression of VEGF and uPA in endometrial cell culture from patients than did CPF. In the current study, not only have we validated Cosín's paper results, but also observed a similar trend when primary cell cultures from OMA were considered.

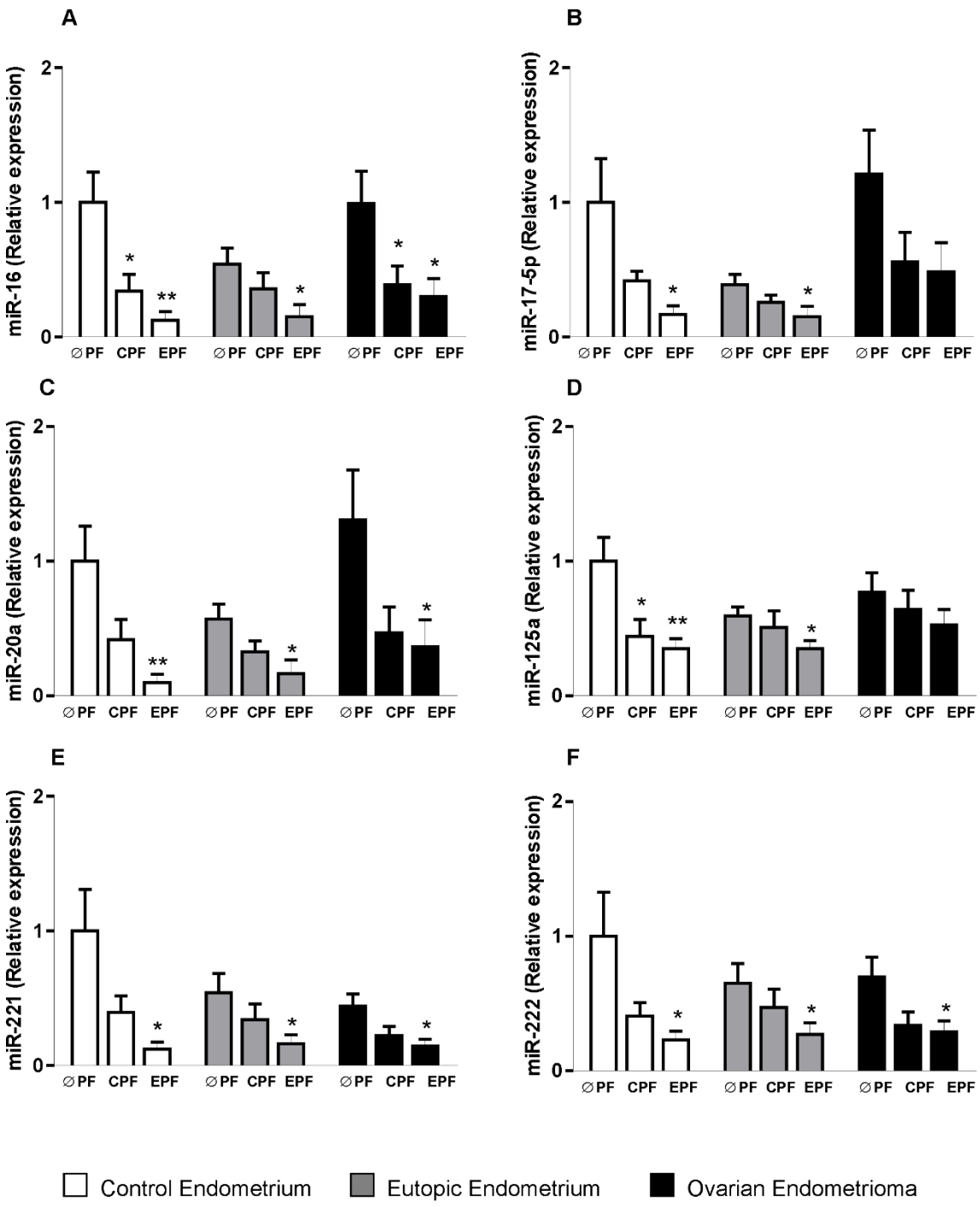


Figure 25. Effects of peritoneal fluid (PF) on the expression of miRNAs in stromal cell cultures of endometriosis and control endometrial tissue and endometriotic of patients. Data are expressed as fold change (mean ± SEM) relative to the mean of control endometrial cell cultures without PF exposure (control endometrium ØPF=1). ØPF: without PF; CPF: control PF; EPF: endometriosis PF.*p <0.05, **p <0.01, ***p <0.001 *vs.* ØPF from the same tissue culture. A) miR-16; B) miR-17-5p; C) miR-20a-5p; D) miR-125a-5p; E) miR-221; F) miR-222. (**Annex 1, Paper 2**).

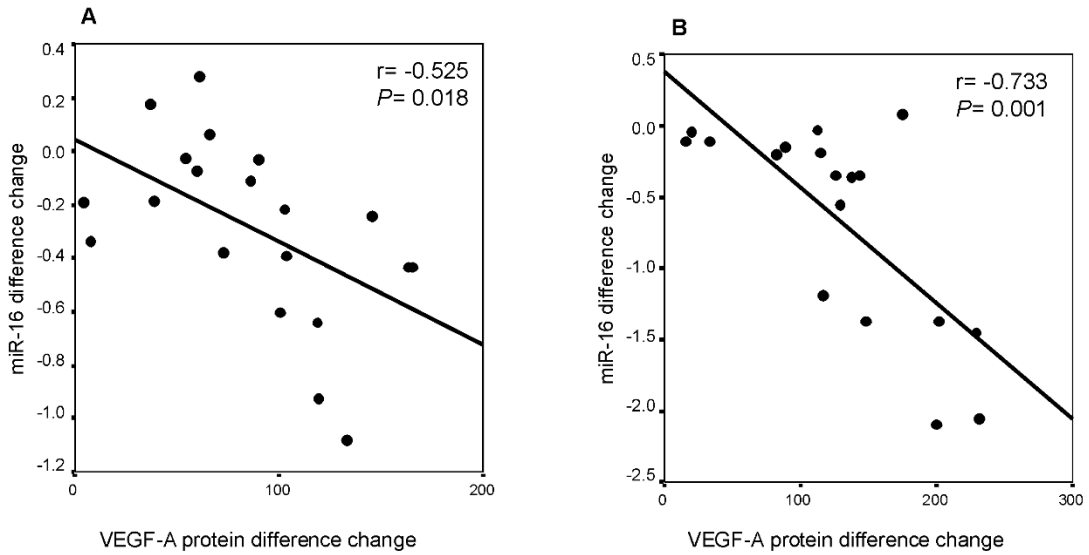


Figure 26. Correlations between VEGF-A protein and angiomiRNA changes after treatment with PFs pools. A) Correlation between changes in miR-16-5p and VEGF-A levels in eutopic cells. B) Correlation between changes in miR-16 and VEGF-A protein levels in ectopic cells. Change: differences between parameter levels with and without PF treatment (**Annex 1, Paper 2**).

With respect to angiogenesis, the angiogenic state of a cell and tissue depends on the so called "angiogenic switch", which refers to the balance of both endogenous activators and inhibitors of angiogenesis within a given biological system. (Lawler and Lawler, 2012). At the light of our results, the imbalance towards an active angiogenic state in patient cell cultures is produced by an increased VEGF-A expression, since levels of TSP-1 do not show statistically significant variations among different experimental conditions. One step further, results also pointed to the contribution of an epigenetic mechanism of post-transcriptional regulation in the expression of VEGF-A, since changes in protein expression were not a consequence of a higher transcriptional rate.

Regarding the expression of the studied miRNAs, PF treatments reduced the expression of miRNAs related to angiogenesis (miR-16-5p, -17-5p, -20a and -125a) and an increase in levels of VEGF-A. These results suggest that miRNAs miR-16, -17-5p, -20a-5p and -125a-5p may act as regulators of VEGF-A in endometrial tissues and ectopic lesions. Furthermore, the fact that *VEGFA* mRNA expression does not vary significantly among treatments is in agreement with an imperfect miRNA:mRNA pairing, which is the most common mechanism of miRNA action in mammals, rendering a mRNA translation repression without mRNA degradation (Bartel, 2004) (**Annex 3, Suppl. Figure 1**).

Additionally, statistical analyses determined a significant inverse correlation between the increase in the expression of this growth factor and miR-16-5p levels, which is consistent with functional studies carried out on endothelial cells (Chamorro-Jorganes et al, 2011) and cancerous (Sun et al, 2013) cells in which VEGF-A was validated as a miR-16 target by functional assays with the reporter luciferase gene.

Our results showed a significant positive correlation between change in levels of miR-17-5p and those of miR-20a after PFs exposure (r= 0.883; p <0.001). This is in agreement with the fact that both miRNAs are encoded in the miR-17-92 cluster, also known as Oncomir-1. Transcription of this gene results in a pri-miRNA encoding 6 mature miRNAs: miR-17-5p, -20a, -18a, -19a, -19b-1 and -92a-1. It is also well-known that c-

Myc induces the expression of Oncomir-1 in several types of tumours, being able to promote proliferation, inhibit differentiation and increase angiogenesis. Although the mechanism through which the miR-17-92 cluster increases angiogenesis remains unknown, two possibilities have been postulated: on the one hand, several authors point to the inhibition of miR-18a and miR-19a mediated expression of the TSR-1 inhibitor (Olive et al, 2010) whereas others suggest an over-expression of both hypoxia-inducible factor (HIF- 1α) (Taguchi et al, 2008) and VEGF-A, (Lei et al, 2009) mediated by the under-expression of miR-17-5p and miR-20a. In addition, both miRNAs have been shown to inhibit the expression of transforming growth factor (TGF-β) (Volinia et al, 2006) and interleukin-8 (IL-8) (Yu et al, 2010). Therefore, the under-expression of these miRNAs may contribute to the local inflammation process and tissue remodelling during the development of the lesion (Kyama et al, 2006). Our results suggest that increased angiogenic activity is mediated by the overexpression of VEGF-A modulated by a reduced inhibitory activity due to the down-regulation of both miRNAs, miR-17-5p and miR-20a, in tissue from patients; taken into account that TSP-1 levels were not significantly modified.

PF represents an important component of the peritoneal microenvironment of endometriotic lesions. However, the PF components capable of inducing deregulation of the miRNAs involved in the regulation of VEGF-A in ectopic lesions remain unknown. PF is a dynamic fluid in a continuous process of changes both in volume, cell composition and cytokines. Moreover, it has been described that the concentration of VEGF-A is higher in EPF (McLaren et al, 1996; Gilabert-Estellés et al, 2007; Cosín et al, 2010) and that PF is capable of inducing the production and secretion of VEGF-A by macrophages (Torry and Torry, 1997).

Thus, our data suggest and involvement of PF in the development of endometrial pathology, enhancing the angiogenic and possibly inflammatory component in endometrial lesions through deregulation of the expression of the studied miRNAs. From a detailed point of view, the angiogenesis process can be divided into two stages: the *activation phase* and the *resolution phase*. In the activation phase, extracellular proteolysis is essential for basement membrane degradation, cell migration and invasion of ECM. Such degradation is mediated by the balance between regulators and effectors. Several components of the ECM are resistant to the action of broad spectrum proteases, but not to the enzymes of the two most relevant families in the degradation of ECM: the plasmin-plasmin activator system and matrix metalloproteases (Pepper 2001; Zorio et al, 2008).

Additionally, it has been described that uPA levels are overexpressed in the endometrium of patients with endometriosis compared to that of control women (Osteen et al, 1996; Cho et al, 2012) and, interestingly, that VEGF-A is capable of inducing expression of uPA (Pepper, 2001). Thus, it seemed reasonable to us to simultaneously study components of the angiogenic and fibrinolytic system in our patients.

In the present study, we have observed an increase in the levels of the fibrinolytic activator uPA in cultured stromal cells after stimulation with both EPF and CPF. In addition, consistent with the literature (Pepper, 2001), we observed a direct significant correlation between the levels of VEGF-A and uPA in CNT culture after exposure to PF pools ($r = 0.717$, $p = 0.019$). These results suggest an important role of the remodelling of the ECM mediated by the fibrinolytic system in the establishment of ectopic lesions.

At the light of the findings highlighted in this chapter, we conclude that our *in vitro* model is suitable to study the angiogenic and fibrinolytic activity involved in the pathophysiology of endometriosis, as well as their regulatory miRNAs. This paved the way for broadening the scope of the miRNA molecules we wished to interrogate, as will be described in the next chapter.

Chapter 3

Chapter 3: Effect of peritoneal fluid on the miRNA expression profiles of endometrialand endometriotic cells from women with endometriosis

Once our *in vitro* model had been validated (Chapter 2), we wished to investigate if the regulation of miRNAs by PF in primary stromal cell cultures could be extrapolated to all known miRNAs. For this purpose, we performed miRNA expression arrays (Affymetrix platform, Array Service, IIS La Fe) in cell cultures exposed to PF pools. As previously stated (see Results section, point 2.1, **Table 8**), samples employed in this study correspond to those analysed in the previous chapter.

3.1. miRNA expression profiles (Affymetrix platform)

The GeneChip miRNA 2.0 Array employed contains probes for 1,105 human mature miRNAs and for 1,105 pre-miRNAs. Profiling of all these RNAs was completed for 3 cell cultures from CNT (control cells), 4 from EUT (eutopic cells) and 3 from OMAs (ectopic cells) treated with PFs from patients (EPF), controls (CPF) and without treatment (ØPF).

Principal Component Analysis (PCA) is a graphical algorithm which groups the samples by similarity in their expression profiles of miRNAs. Thus, the PCA revealed that control cells treated with CPF showed no modification in the miRNA expression pattern in comparison to untreated cells. Nevertheless, the miRNA expression was different in response to EPF **(Fig. 27A**). In contrast to control cells, eutopic and ectopic cells responded to EPF and CPF in a different way in terms of miRNA expression (**Fig. 27B-C**).

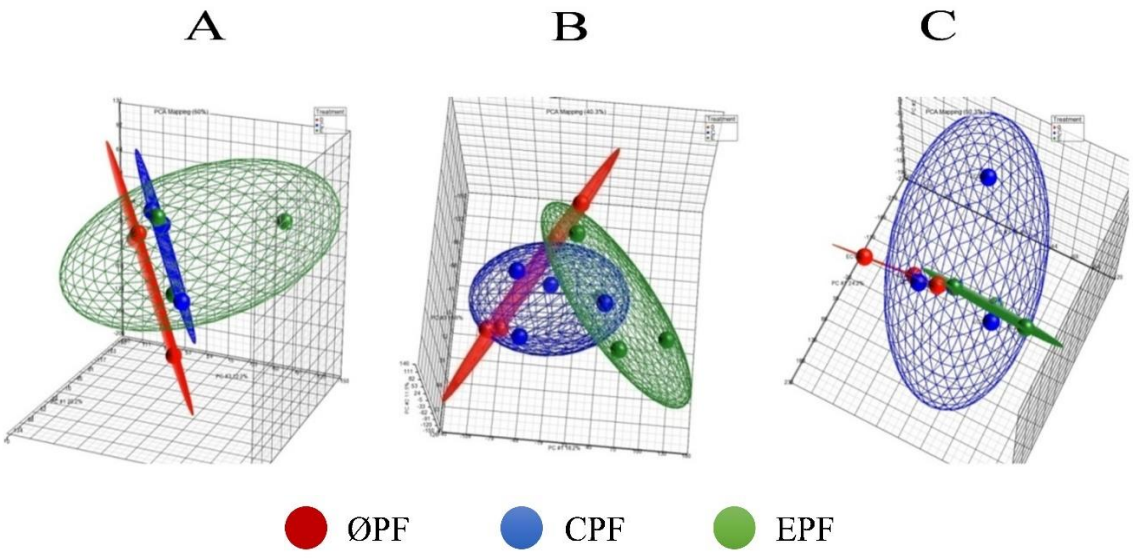


Figure 27: Principal component analysis (PCA) performed from miRNA expression arrays. A) control cells, B) eutopic cells, C) ectopic cells. ØPF: without any treatment; CPF: control PF treatment; EPF: endometriosis PF treatment (**Annex 1, Paper 3**).

Volcano plots are a type of scatter-plot used to quickly identify changes in large data sets. They show p-value on the ordinate axe, commonly in logarithmic scale, *vs*. log2 of fold-change on the abscissas axe. In our graphs, miRNA significantly differently expressed are coloured in blue, defining the vertical lines ±2-fold change (FC). Volcano plots from ANOVA test (**Fig. 28**) revealed that the major difference in miRNA expression was observed in eutopic cells after EPF treatment (**Fig. 28E** and **28H**). Moreover, it should be highlighted that the majority of these miRNAs were down-regulated in response to EPF. The comparison between the response to EPF and to ØPF showed that eutopic cells presented the highest number of miRNAs significantly deregulated (±2-FC, $p < 0.05$) (**Fig. 28E**).

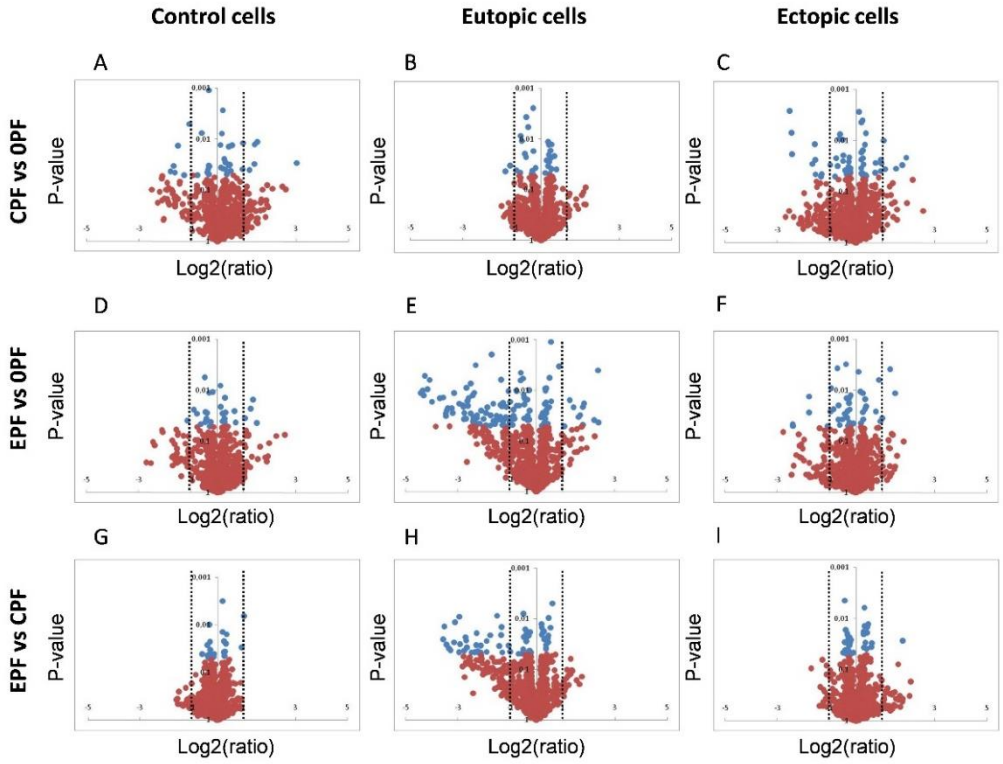


Figure 28. Volcano plots representing miRNA differently expressed in control, eutopic or ectopic cells in response to different treatments. Note the logarithmic scale in the ordinate axis. ØPF: without any treatment; CPF: control PF treatment; EPF: endometriosis PF treatment (**Annex 1, Paper 3**).

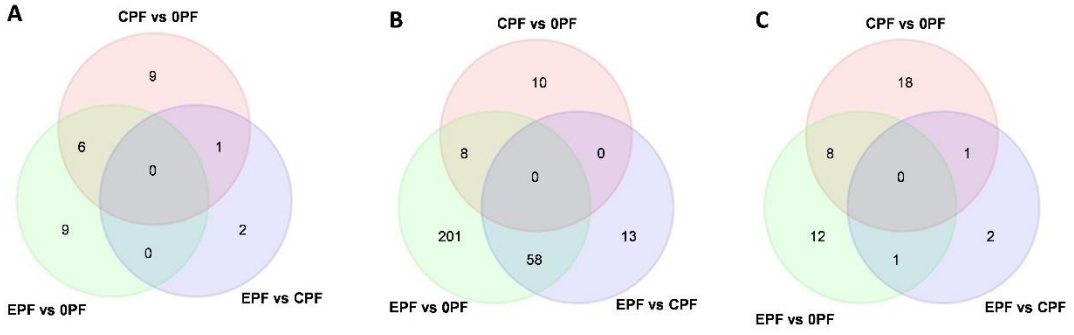


Figure 29. Venn diagrams representing the number of miRNAs deregulated in each experimental condition. A) Control cells; B) eutopic cells; C) ectopic cells. ØPF: without any treatment; CPF: control PF treatment; EPF: endometriosis PF treatment. Student's t-test (**Annex 1, Paper 3**).

Venn diagrams (**Fig. 29**) representing all the differentially expressed (p <0.05) human miRNA probes in the array showed that the major number of deregulated miRNAs were obtained in eutopic cells when treated with EPF. This experimental condition modified the number of differentially expressed miRNAs >12-times when compared to control and ectopic cells.

Among the 267 miRNAs that were modified in response to EPF compared to ØPF in eutopic cells, 82 corresponded to mature miRNAs (72 down-regulated and 10 up-regulated) (±2-FC; p <0.05) (**Annex 2, Suppl. Table 7**). After the *in silico* study of target genes for these differentially expressed miRNAs, we selected 9 miRNAs related to angiogenesis (miR-16-5p, miR-21-5p, miR-29c-3p, miR-106b-5p, miR-130a-5p, miR-149-5p, miR-185-5p, miR-195-5p, miR-424-5p) for validation by qRT–PCR in a larger number of experiments. Of them, 8 were down-regulated in the expression arrays (namely miR-16-5p, miR-21-5p, miR-29c-3p, miR-106b-5p, miR-130a-5p, miR-185-5p, miR-195-5p, miR-424-5p), and miR-149-5p was up-regulated (**Table 9**). Finally, endogenous snoRNA U6 was quantified for normalization purposes.

Table 9. miRNA microarray expression and targets of miRNA selected for the PCR experiments (**Annex 1, Paper 3**).

			Eutopic cells response to EPF vs ØPF		
miRNA (v.15)[a]	**miRNA (v.20)[b]**	**miRNA sequence 5' → 3'**	**Fold change**	***p*-value**	**Target**
miR-16	miR-16-5p	U**AGCAGCA**CGUAAAUAUUGGCG	–9.96870	0.04321	VEGFA, EGFR2, BCL2, FGFR1, COX2
miR-21	miR-21-5p	U**AGCUUAU**CAGACUGAUGUUGA	-16.96419	0.00620	TIMP3, TGFb2, SERPINB5, VEGFA, BCL2, EGFR, MMP2, HIF1a, MMP8, TGFb, TGFBR1, THBS1, TNFRSF11B
miR-29	miR-29c-3p	U**AGCACCA**UUUGAAAUCGGUUA	-2.38095	0.01301	VEGFA, PDGFB-C, THSD4, SERBP1,ADAMTS2,5-7, 9, 17-19
miR-106b	miR-106b-5p	U**AAAGUGC**UGACAGUGCAGAU	-20.34633	0.01002	TGFBR2, MMP2, THSD3, CCNG2, ADAM9, IL8, MMP24, COL4A3, CCND1, TIMP2, CCND2,COL19A, FGF4, VEGFA
miR-130a	miR-103a-3p	C**AGUGCAA**UGUUAAAAGGGCAU	-19.25180	0.00517	SERPINE1, COL4A1, IL6R, COL4A5, VEGFA,COL1A2, SERPINB7, FAS (TNFR superfamily)
miR-149	miR-149-5p	U**CUGGCUC**CGUGUCUUCACUCCC	3.43946	0.04766	GPC1, FGFR1[c], EDNRA, TNFRSF19
miR-185	miR-185-5p	U**GGAGAGA**AAGGCAGUUCCUGA	-19.84907	0.01248	VEGFA, THSD7A, CLDN11, IL17R, HIF3a, EDA2R
miR-195	miR-195-5p	U**AGCAGCAC**AGAAAUAUUGGC	-4.99004	0.00318	COL12A1, CDCA4, BCL2L2, VEGFA, CLDN12, CCND1, SERBP1, DICER1, ADAMTS5, GHR, CLDN2, ESRRA, ESRRG, ADAMTS1
miR-424	miR-424-5p	C**AGCAGCA**AUUCAUGUUUUGAA	-2.03838	0.04712	VEGFA, IL1, FGF2

ADAMTS2, 5-7, 9, 17-19:ADAMmetallopeptidase with thrombospondin type 1 motif, 2, 5-7, 9, 17-19; BCL2: B-cell lymphoma 2; BCL2L2: BCL2-like 2;CCND1: cyclin D1; CCND2: cyclin D2; CCNG2: cyclin G2; CDCA4: cell division cycle associated 4; CLDN11: claudin 11; CLDN12: claudin 12; COL1A2: collagen, type I, alpha 2; COL4A1: collagen, type IV, alpha 1;COL4A3: collagen, type IV, alpha 3; COL4A5: collagen, type IV, alpha 5; COL12A1: collagen, type XII, alpha 1; COL19A: collagen, type IXX, alpha;COX2: cyclooxygenase 2; DICER1: dicer 1, ribonuclease type III; EDA2R: ectodysplasin A2 receptor; EDNRA: endothelin receptor type A; EGFR2: epidermal growth factor receptor 2; ESRRA: estrogen-related receptor alpha; ESRRG: estrogen-related receptor gamma; FAS (TNFR superfamily): Fas cell surface death receptor; FGF2: fibroblast growth factor 2; FGF4: fibroblast growth factor 4; FGFR1: fibroblast growth factor receptor 1; GHR: growth hormone receptor; GPC1: glypican 1; HIF1a-3a: hipoxia inducible factor 1-3, alpha subunit; IL1: interleukin 1; IL6R: interleukin 6 receptor; IL8: interleukin 8; IL17R: interleukin 17 receptor; MMP2: matrix metalloproteinase-2; MMP8: matrix metalloproteinase-8; MMP24: matrix metalloproteinase-24; PDGFB-C: platelet-derived growth factor polypeptide-C; SERPINE1: plasminogen activator inhibitor type 1; SERPINB5-7: serpin peptidase inhibitor, clade B, member 5-7; TGFb2: transforming growth factor beta 2; TGFBR1: transforming growth factor, beta receptor 1; TGFBR2: transforming growth factor, beta receptor 2;THBS1: thrombospondin 1; THSD3: thrombospondin, type 1, domain containing 3; THSD4: thrombospondin, type 1, domain containing 4; THSD7A: thrombospondin, type I, domain containing 7A; TIMP3-2: tissue inhibitor of metalloproteinases-3-2; TNFRSF11B: tumor necrosis factor receptor superfamily, member 11b; TNFRSF19: tumor necrosis factor receptor superfamily, member 19; VEGFA: vascular endothelial growth factor. EPF, endometriotic peritoneal fluid; ØPF, without peritoneal fluid.In the miRNA sequence, seed sequence is highlighted in bold.

[a]Referred to miRBase database release (version 16).

[b]Referred to miRBase database release (version 20). miRNAs are named in microarray according to miRBase version 16. However, the current classification is referred to miRBase 20 release.

[c](Chamorro-Jorganes et al, 2014).

*ANOVA.

It is noteworthy to mention that, with the only exception of miR-125a-5p (FC= 1.74 *p*= 0.22), all of the miRNAs considered for the study in Chapter 1 were also down-regulated in the array of miRNA expression. This can be checked in (**Annex 2, Suppl. Table 2)** with the exception of miR-222, as this did not reach statistical significance (FC = -1.06; *p*= 0.68).

Additionally, as we hypothesized in the previous chapter, we observed a down-regulation of all the miRNAs encoded in the cluster 17-92. This seems to indicate that EPF is able to repress the expression of this polycistronic gene.

3.2. Validation by qRT–PCR

With respect to the down-regulated miRNAs selected for validation by qRT-PCR, all of them showed statistically significant lower levels after EPF treatment in primary cell cultures from eutopic endometrium from patients (**Fig. 30A–E and 30G-I**). Nevertheless, miRNA-149-5p did not showincreased levels after EPF treatment, as the arrays results had shown (**Fig. 30F**).

Additionally, miR-16-5p and miR-424-5p showed lower levels after CPF and EPF treatments in control cells. Ectopic cells reduced the expression of miR-16-5p, miR-29c-3p, miR-106b-5p, miR-130a-5p and miR-185-5p in the presence of both PF pools, being variation in miR-16-5p levels the only statistically significant change.

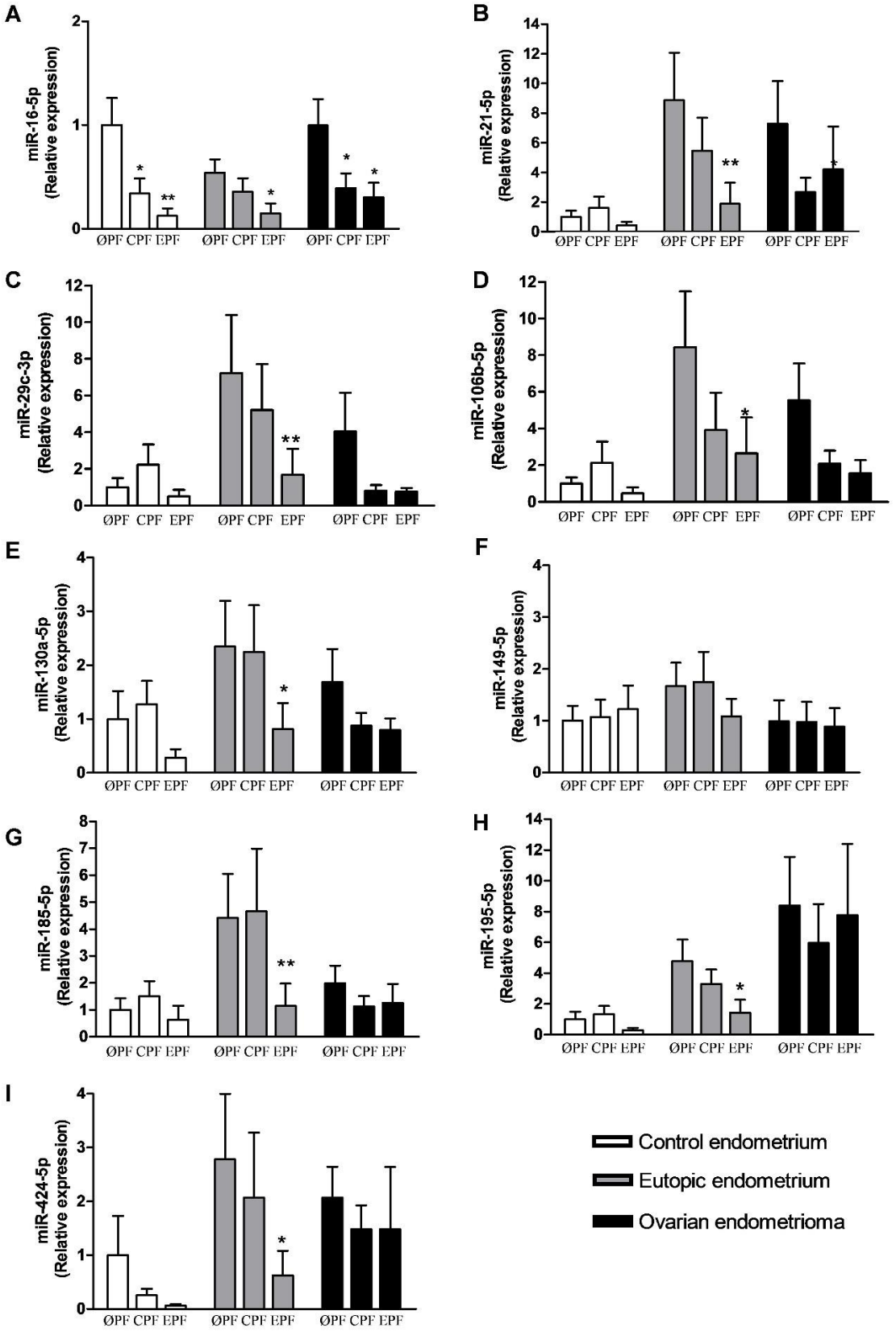


Figure 30. miRNAs validated by qRT-PCR in control (n=8), eutopic (n=11) and ectopic (n=11) cells. ØPF: without PF treatment; CPF: control PF treatment; EPF: endometriosis PF treatment. **p* <0.05, ***p* <0.01, *vs.* ØPF from the same tissue culture. A) miR-16-5p; B) miR-21-5p; C) miR-29c-3p; D) miR-106b-5p; E) miR-130a-5p; F) miR-149-5p; G) miR-185-5p; H) miR-195-5p; I) miR-424-5p (**Annex 1, Paper 3**).

3.3. Functional experiments using mimics

As previously described, miR-16-5p, miR-29c-3p and miR-424-5p were significantly down-regulatedin eutopic cells after EPF treatment in the validation phase.

In silico analysis revealed that all three miRNAs could regulate the translation of VEGF-A (**Table 9**). With the aim to specifically investigate the regulation of VEGF-A expression by these three miRNAs, we transfected primary cell cultures from control and patient endometrium with mimics of miR-16-5p, miR-29c-3p and miR-424-5p (**Fig. 31**). Additionally, we employed the established EA.hy926 endothelial cell line as experimental control.

Firstly, we focused on optimizing the dose for the subsequent experiments. To this end, we transfected cultured control cells with increasing concentrations of either the scramble RNA or miRNA mimics (20 nM, 50 nM and 100 nM). We observed a dose-dependent reduction of VEGF-A protein levels in cell supernatants with increasing dosis of mimics. Thus, we chose 100 nM as the optimal dosis for subsequent experiments.

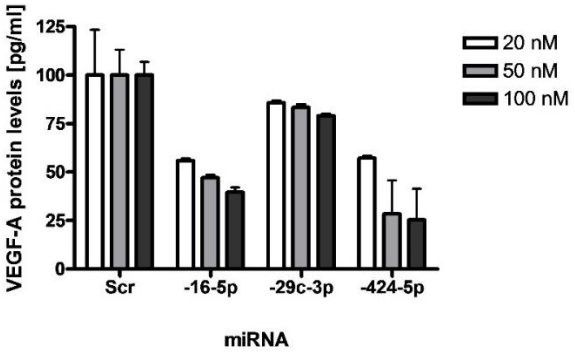


Figure 31. VEGF-A protein levels after control cell transfection with increasing concentrations (20 nM, 50 nM of scramble RNA or miR-16-5p, miR-29c-3p and miR-424-5p mimics, 100 nM), measured by ELISA in cell supernatants (**Annex 1, Paper 3**).

We next wanted to assess the possible effect of endogenous miRNAs on mimic transfections. To this end, we quantified miRNAs levels by qRT–PCR after mimic transfection (**Fig. 32**). Results validated the effect of exogenous synthetic miRNAs used in the functional studies. As can be observed, after miRNAs mimic transfections the contribution of endogenous miRNAs levels to total amount of a given miRNA was negligible.

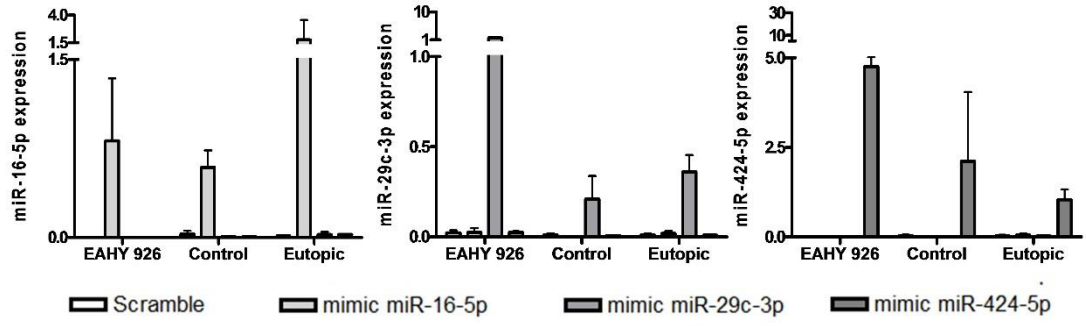


Figure 32. miRNA levels quantified by qRT-PCR after mimic transfections (100 nM) of miR-16-5p, miR-29c-3p, miR-424-5p and scramble RNA (**Annex 1, Paper 3**).

In the EA.hy926 cell line, transfection with miR-16-5p, miR-29c-3p or miR-424-5p mimics induced a reduction of 63±11%, 76±0.9% and 79±0.9% (p <0.01) in VEGF-A expression when compared to scrambled RNA, respectively (**Fig. 33A** and **33D**). When performing the same transfections in primary cell cultures from control and patient endometria, VEGF-A expression was reduced by 79±20% (p =0.12), 90±0.2% and 90±0.2% (p <0.001) in endometrial cells from women without the disease, when compared to scrambled miRNA (**Fig. 33B** and **33D**) and 96% (p <0.001), 79% and 78% (p <0.01) in patient endometrial cells, respectively (**Fig. 33C** and **33D**).

VEGFA mRNA levels were quantified after transfections, showing no statistically significant modifications in any ofthe studied cell types (**Fig. 33E**).

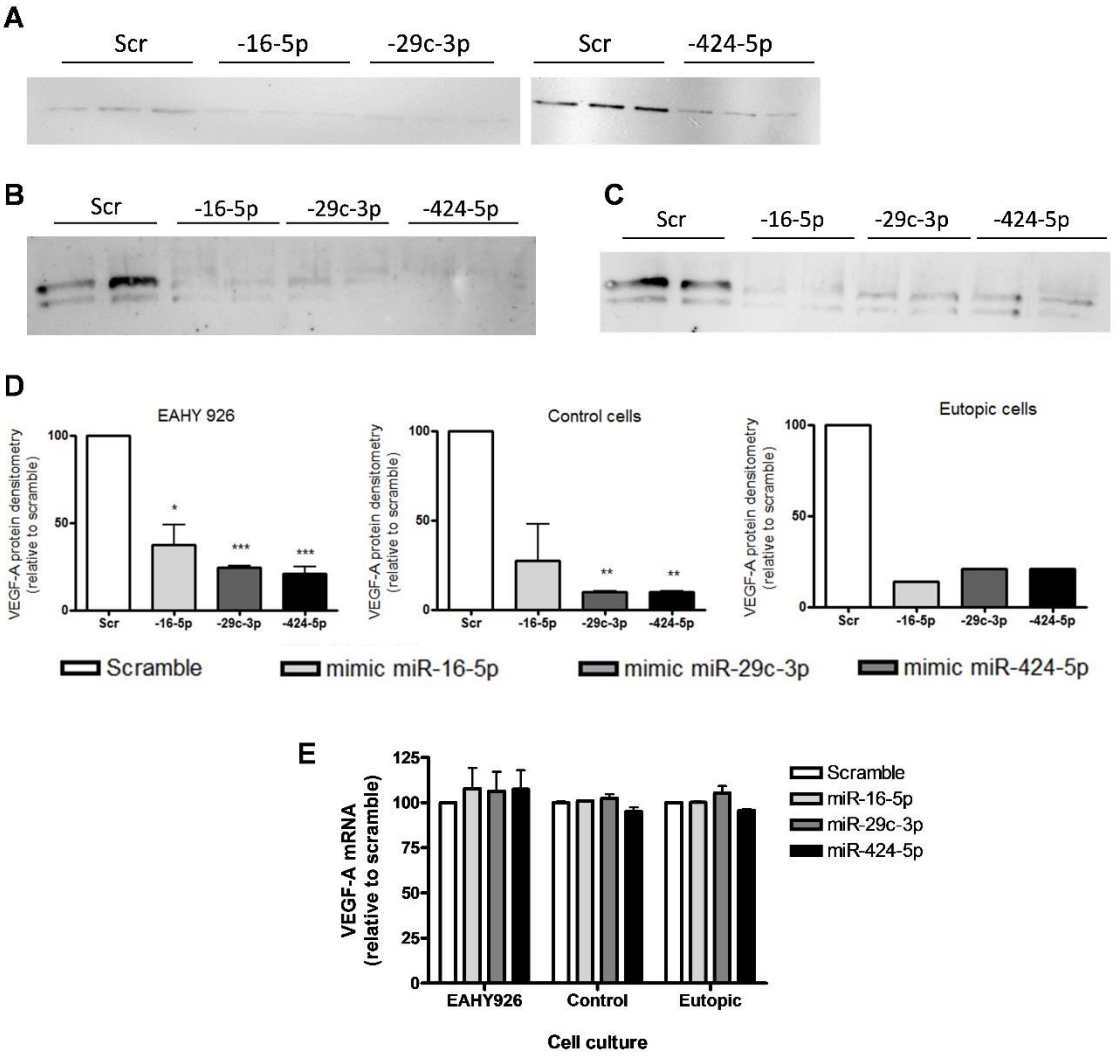


Figure 33. *In vitro* studies for miRNA mimics transfection. A)–C) Representative western blots for VEGF-A after transfection of cells A) EA.hy926 cell line; B) control cells; C) eutopic cells with scramble RNA or miR-16-5p, miR-29c-3p and miR-424-5p mimics (100 nM) for 48 h. D) Densitometric analysis of VEGF-A extracellular expression. E) VEGF-A protein levels after control cell transfection with mimics (20 nM, 50 nM and 100 nM) measured by ELISA. Scr: scramble RNA. All experiments were performed in triplicate (n=3). ANOVA test. *p <0.05, **p <0.01 and ***p <0.001(modified from **Annex 1, Paper 3**).

3.4. Validation of miRNA:VEGF-A mRNA interaction

To confirm that miR-29c-3p directly modulate VEGF-A expression, *VEGFA* 3′-UTR was cloned downstream from the firefly luciferase open reading frame. Both the wild-type (WT) reporter construct and the *VEGFA* 3'-UTR construct with themiR-29c-3p binding site mutated (MUT) were co-transfected in different experiments (**Fig. 34A**) in the HCT116-Dicer KO cell line, with the scramble RNA or a miR-29c-3p mimic.

Our results showed that the relative luciferase activity was significantly decreased in cells co-transfected with the wild-type construct and miR-29c-3p (62±8%, p <0.001), indicating the complementary binding to the *VEGFA* 3'-UTR. However, this inhibition was not observed when co-transfection was performed with the vector containing the specifically mutated 3′UTR of *VEGFA* (**Fig. 34B**), suggesting that *VEGFA* is a specific target of miR-29c-3p.

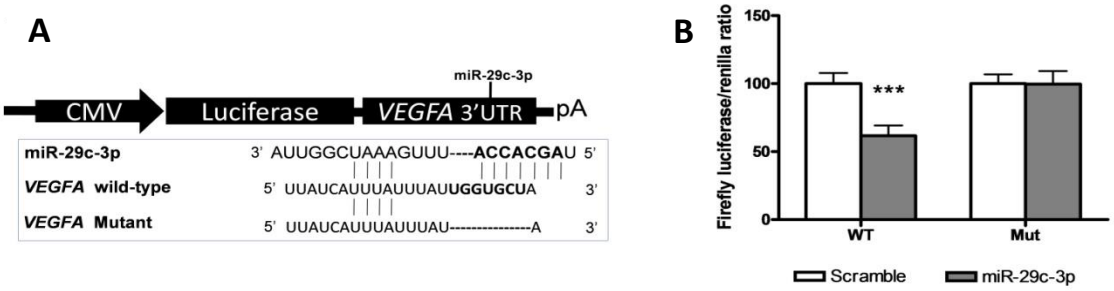


Figure 34. Luciferase experiments to validate *VEGFA* mRNA:miR-29c-3p interaction. A) *VEGFA* mRNA levels after mimic transfection (100 nM). B) Schematic representation of miR-29c-3p predicted target site in *VEGFA* 3′-UTR. Complementarities between the seed region (seven nucleotides) of miR-29c-3p and 3′-UTR of *VEGFA* mRNA target site are shown. HCT116, the dicer KO cell line, was co-transfected with scramble RNA or miR-29c-3p mimic and pMIR-*VEGFA*-3′ UTR wild-type (WT) or mutated (Mut). All experiments were performed in triplicate (n=3). ANOVA test. *p <0.05, **p <0.01 and ***p <0.001 (modified from **Annex 1, Paper 3**).

3.5. Discussion

In the present study, we observed that PF from patients modified themiRNA expression profile in endometrial stromal cells from women with endometriosis, including miRNAs involved in angiogenesis. miR-16-5p, miR-29c-3p and miR-424-5p, well-known angio-miRNAs, presented significant reductions when cells from patients were treated with EPF. Because of that, we performed functional studies employing mimics for these miRNAs in order to validate their potential role as regulators of VEGF-A protein expression. Data pointed that these miRNAs regulateVEGF-A translation not only in the EA.hy926 cell line but also in cells from endometrial tissues from women with and without endometriosis.

Angiogenesis plays an important role in multiple physiological and pathological processes including gynaecological diseases like endometriosis. Several miRNAs can control the expression of VEGF-A. miR-29c-3p is a multifunctional miRNA implicated in several processes, including extracellular remodelling and angiogenesis, and can contribute to the formation of endometriotic lesions in patients with endometriosis (Braza-Boïls et al, 2014). A study performed in rats (Yang et al, 2013) demonstrated that

VEGF-A is a direct target of miR-29a and miR-29c and these miRNAs suppressed endogenous VEGF-A expression *in vitro*. In the present study, we have observed that the transfection of miR-29c-3p in endometrial and endometriotic cells from patients with endometriosis significantly decreased VEGF-A protein expression. Furthermore, luciferase experiments indicated that VEGF-A is a direct target of miR-29c-3p also in humans.

Both miR-16-5p and miR-424-5p have the same 'seed sequence' (see table 9), and thus they target the same nucleotide sequence in the VEGFA mRNA, which implies that both miRNAs can share most of their target genes. In the present work, we observed a significant reduction in VEGF-A protein expression in primary cell cultures from controls and patients' endometria after transfection with miR-16-5p or miR-424-5p mimics. However, *VEGFA* mRNA expression after mimic transfection was not significantly modified. In our case, this indicates that miRNAs miR-16-5p, miR-29c-3p and miR-424-5p mainly inhibit VEGF-A translation without degrading *VEGFA* mRNA. This is in agreement with the aforementioned mechanisms of regulation with imperfect miRNA:mRNA pairing (Chapter 2) (see **Annex 3, Suppl. Fig. 1** for miR-16:VEGFA and miR-424-5p:VEGFA and **Annex 3, Suppl. Fig.2** for miR-29c-3p), this is, the decrease in protein levels without significant modification of mRNA levels (Bartel, 2004).

Indeed, in a previous study (Chapter 2 and **Annex 1, Paper 2**), we investigated the influence of PF from women with and without endometriosis on the expression of six miRNAs, including miR-16-5p, which modulate angiogenesis, as well asseveral angiogenic and proteolytic factors in endometrial and endometriotic cell cultures. We found a significant correlation between the decrease in miR-16-5p and the increase in VEGF-A protein, but not mRNA, in response to PF exposure in endometrial and endometriotic cell cultures. In the current study, we evaluated all the known miRNAs by means of a GeneChip miRNA array (Affymetrix Platform). With the only exception of miR-125a-5p (FC= 1.74, p= 0.22), all of the miRNAs considered for the study in Chapter 2 were also down-regulated in the array of miRNA expression (**Annex 2, Suppl. Table 7**), enhancing the robustness of our results.

In a previous report, Chamorro-Jorganes and co-workers (Chamorro-Jorganes et al, 2011) investigated the role of miR-16-5p and miR-424-5p in the angiogenic activity of endothelial cells, andshowed that both miRNAs directly targeted VEGFA mRNA. These results are in agreement with results obtained in the present study, in which we observed that miR-16-5p and miR-424-5p can regulate VEGF-A protein levels in endometrial and endometriotic cells, as occurred in endothelial cells.

We previously suggested (Chapter 1 and **Annex 1, Paper 1**), that miR-424-5p contributed, at least in part, to the higher VEGF-A levels observed in the endometrium from patients with endometriosis. Other authors indicated that miR-424-5p targets VEGF-A and plays an important role in down-regulating the angiogenic activity of this protein (Wang and Olson, 2009; Chamorro-Jorganes et al, 2011). Moreover, Nakashima and co-workers (Nakashima et al, 2010) reported that down-regulation of mir-424 can contribute to the abnormal angiogenesis in senile haemangioma.

In conclusion, PF from patients modified the miRNA expression profile in endometrial cells from women with endometriosis. Functional studies employing mimics for miR-16-5p, miR-29c-3p and miR-424-5p suggested that these miRNAs regulate VEGF-A

translation not only in EA.hy926 cells but also in primary stromal cells from endometrium from patients with endometriosis and control women. These promising results really improve the body of knowledge about endometriosis pathogenesis that could open up new therapeutic strategies for the treatment of this condition through the use of miRNAs, as is being conducted in other pathologies (van der Ree et al, 2016; Beg et al, 2017).

Chapter 4

Chapter 4. Epigenomic landscape in endometriosis. Analysis of the genome-wide DNA methylation

4.1. Clinical characteristics of patients included in the study

Clinical characteristics of the cohort of study are shown in **Table 10**.

We initially evaluated whether our case and control cohorts were similar or comparable in terms of several clinical variables by using biostatistics tools, hereafter described. In this sense, our cohort consisted on 13 Caucasian women with endometriosis (stage III-IV) (mean age 35.2 years, range 24-42) and 11 Caucasian women without endometriosis (mean age 36.6 years, range 30-44). As several studies had shed light into the influence of age on the methylation status (Issa et al, 2003; Fraga and Esteller, 2007), we aimed to determine if our case-control population were comparable, finding no statistically significant differences between patients and control women in terms of age (p =0.483).

Secondly, regarding the phase of the menstrual cycle, Houshdaran and co-workers (Houshdaran et al, 2014) reported its influence over DNA methylation patterns in endometria from women without endometriosis. Thus, to control this variable, we included in our experimental design samples in proliferative, secretory and menstrual phases. For the endometriosis cohort, 5 (38.4%) women were in the proliferative phase, 4 (30.8%) in the secretory phase and 4 (30.8%) in the menstrual phase of the cycle. In parallel, 4 (36.4%) control women were in the proliferative phase, 4 (36.4%) in the secretory phase and 3 (27.2%) in the menstrual phase of the cycle.

Another clinical factor could be endometriosis stage; all patients included presented stages III-IV. Since it remains uncertain whether the three main types of endometriotic lesions (OMA, PI and RVN) are variants of the same pathologic process or caused by different mechanisms (Nisolle and Donnez, 1997; Bulun, 2009) and aberrant DNA methylation patterns might be involved in the aetiology of endometriosis, we included all patients having OMA lesions, albeit two of them presented an extra lesion (PI and RVN, respectively).

Table 10. Clinical characteristics of patients included in the study.

	CONTROL WOMEN (n = 11)	**ENDOMETRIOSIS** (n = 13)
AGE (YEARS; MEAN± SEM)	36.6 ± 4.5	35.2 ± 5.0
RANGE (YEARS)	[30 - 44]	[24 - 42]
CYCLE PHASE		
• *PROLIFERATIVE*	n = 4 (36.4%)	n = 5 (38.4%)
• *SECRETORY*	n = 4 (36.4%)	n = 4 (30.8%)
• *MENSTRUAL*	n = 3 (27.2%)	n = 3 (30.8%)
ENDOMETRIOTIC LESIONS		
• *OMA*	NA	n = 13 (100%)
• *PI*	NA	n = 1 (7.7%)
• *RVN*	NA	n = 1 (7.7%)
STAGE		
• *I-II*	NA	n=0 (0%)
• *III-IV*	NA	n=13 (100%)

NA: not applicable

4.2. DNA methylation profiles determined by the methylationEPIC BeadChip

The MethylationEPIC BeadChip microarray covers over 850,000 CpG methylation sites (850K) with >90% of the 450K sites plus an additional 333,265 CpGs located in enhancer regions identified by the ENCODE and FANTOM5 projects (Moran et al, 2016).

Therefore, taking advantage of this state of the art technology, we have obtained the whole-genome DNA methylation profiling of 11 endometria from control women (3 in proliferative phase, 4 in secretory phase and 3 in menstrual phase) and 13 endometria from patients with endometriosis (5 in proliferative phase, 4 in secretory phase and 4 in menstrual phase). Raw data generated were pre-filtered, normalized and prepared for following analyses as described in Material and Methods section.

4.2.1. Unsupervised hierarchical clustering of methylation levels of 5000 random selected CpGs

Firstly, we were interested in evaluating whether genome wide distribution of DNA methylation segregated control and endometriosis patients. However, using an unsupervised hierarchical clustering of methylation levels of 5000 random selected CpGs we observed neither segregation by disease status (patients *vs.* control) nor by cycle phase (proliferative, secretory or menstrual). This result suggests that differences, if any, might concern a reduced number of specific CpGs (**Fig. 35**).

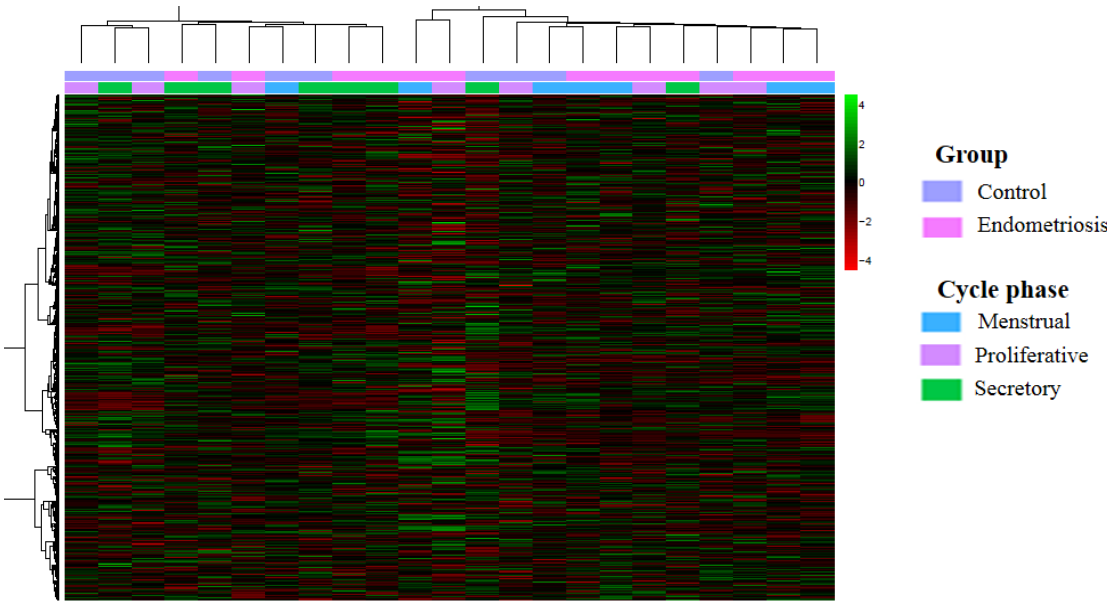


Figure 35. Unsupervised hierarchical clustering of endometrial samples from patients with endometriosis and control women analysed by the DNA MethylationEPIC Beadchip array (Illumina).

4.2.2. Scatter plots of 866,836 CpGs

To confirm the similarity in DNA methylation profiles between groups, scatter plots and correlation analysis were carried out using all valid CpGs (866,836 CpGs) (**Fig. 36**). These graphical plots use Cartesian coordinates to display each CpG-associated β-values for patients (y-axis) and control women (x-axis). As can be observed, there is a high degree of correlation for patient and control β-values for each CpG (see Pearson coefficients for each comparison). Same results can be observed when analysing by the phase of the menstrual cycle (**Fig. 36A-C**).

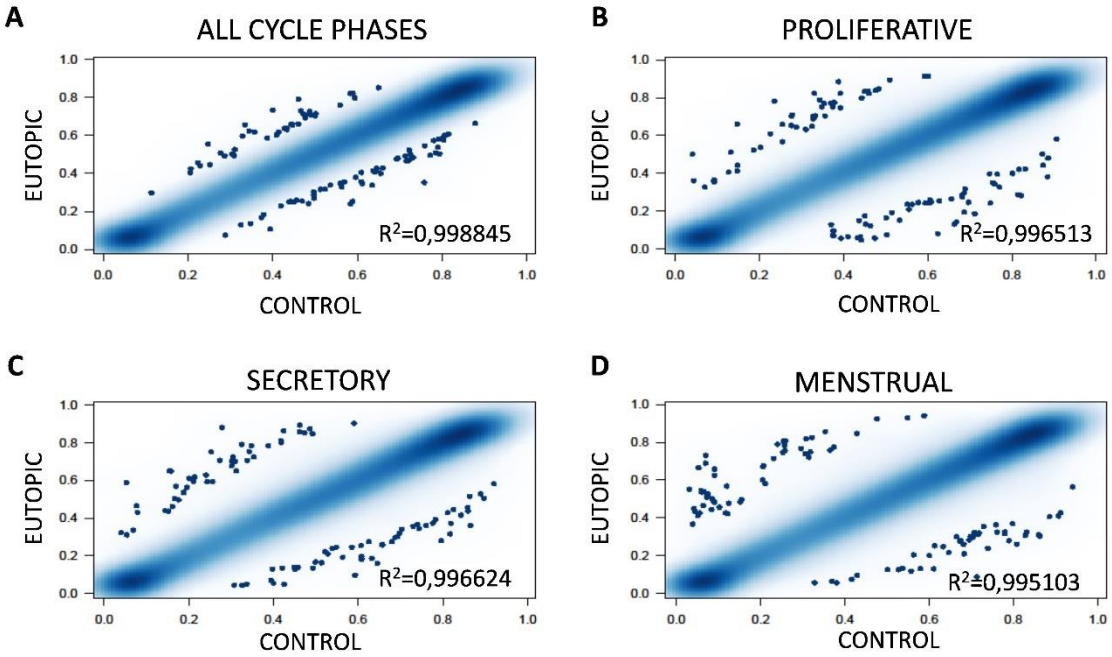


Figure 36. Smooth scatter plots reporting methylation levels of 866,836 CpGs between samples from patients with endometriosis and control women. A) regardless of the cycle phase, or comparing B) proliferative phase C) secretory phase and D) menstrual phase. R^2= Pearson coefficient.

4.2.3. Analysis of differentially methylated regions (DMR)

One step further, we wanted to determine if there may exist, rather than single CpG methylation changes, differentially methylated regions (DMR) in the genome that could distinguish the EUT *vs.* the CNT methylome. DMR calculation was conducted as described (see Material and Methods). Nevertheless, any significant DMR was found across our cohorts of study (data not shown).

4.2.4. Statistic model distinguishing patients with endometriosis from control women

Whole-genome DNA methylation patterns were not enable to differentiate between samples from patients with endometriosis and control women. However, to define the existence of potential specific elements of distinction in these two groups we used a

supervised elastic net-penalized logistic regression model. This statistical analysis determined 7 CpGs which together provided the highest discrimination power between patient and control women's samples (**Table 11** and **Fig. 37**).

Three out of the seven CpGs (namely CpG11496778, CpG01378439 and CpG04296894) have not been assigned as belonging to any known gene. Conversely, the remaining 4 CpGs were associated with LDL receptor related protein 5 (*LRP5*), Ssemaphorin4F (*SEMA4F*), myosin light chain 12B (*MYL12B*) and phosphoinositide-3-kinase adaptor protein 1 (*PIK3AP1*), any of them previously associated with endometriosis. Notably, Δβ for all seven CpGs was rather small, regardless of the phase of the menstrual cycle considered **(Fig. 38**). Thus, three of these CpGs were hypermethylated (those associated with *LRP5, PIK3AP1* and CpG04296894; β-value ≥0.8), one was intermediately methylated (CpG01378439; 0.2≤ β-values ≤0.8) and three were hypomethylated (those associated with *MYL12B, SEMA4F* and CpG11496778; β-values ≤0.2).

Table 11. Description of the 7 CpGs included in the model determined by elastic net analysis.

Probe ID	Genomic localization	Gene
cg01378439	NA	NA
cg01907723	Chr10: 98353069-98480279	PIK3AP1
cg04296894	NA	NA
cg11496778	NA	NA
cg14839905	Chr11: 68181086-68181289	LRP5
cg15270558	Chr2: 74881359-74882019	SEMA4F
cg27114094	Chr18: 3261708-3262671	MYL12B

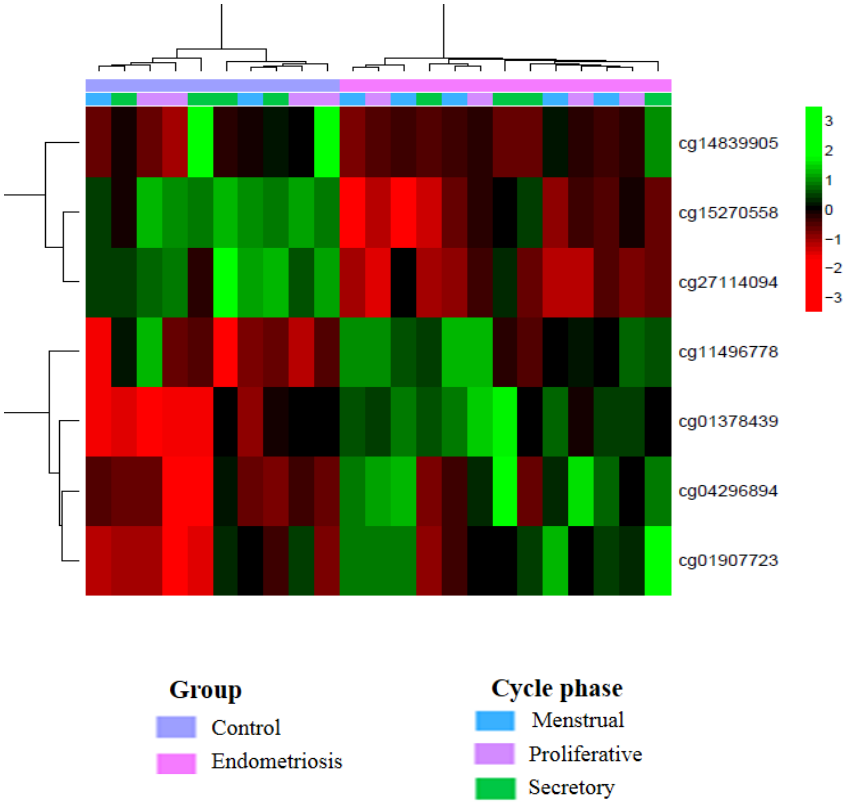


Figure 37. Heat map of the 7 CpGs predicted by the model.

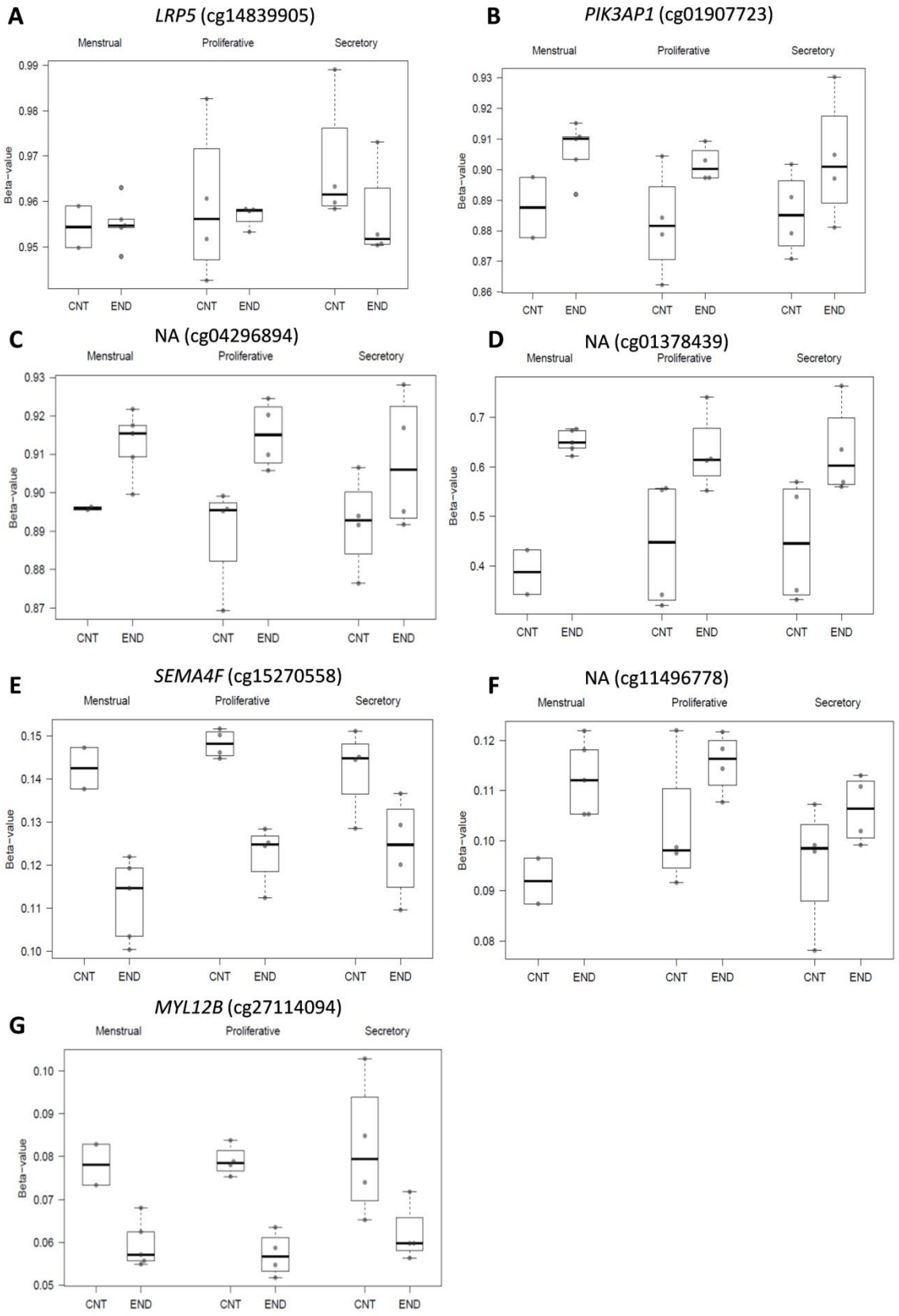


Figure 38. Methylation levels by phase of the menstrual cycle and disease-status of the 7 CpGs predicted by the model which can differentiate patients with endometriosis from controls. CNT: control women's endometria; END: endometriosis patients' endometria.

4.3. Characterization of endometrial genome-wide methylome

Due to the importance of the endometrium in the implantation process and the singularity of the tissue in terms of cyclical changes in gene expression under hormonal influence, we aimed to provide a detailed description of genome wide DNA methylation profile of this tissue. 202,007 out of the 866,836 CpGs analysed (23.3%) were found to be hypomethylated (β ≤0.2), whereas 214,930 (24.8%) were hypermethylated CpGs (β ≥0.8) in the endometrial tissues analysed (**Fig. 39**).

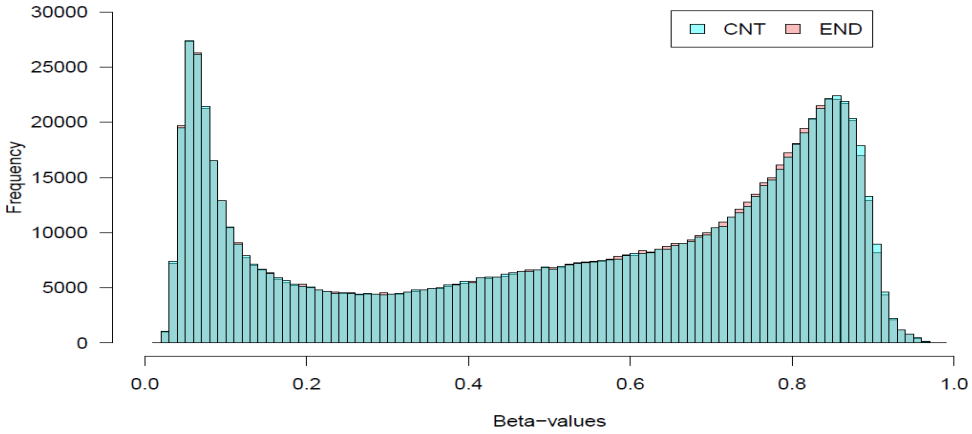


Figure 39. Density plot representing frequency of CpGs with a specific methylation level (represented as β-value). As can be seen, curves for patients and controls are overlapped. CNT: control women's endometria; END: endometriosis patients' endometria.

4.3.1. Genomic distribution of differentially methylated GpGs in the human endometrium

Genomic distribution of over 866,836 CpGs in gene bodies, intergenic regions and promoter regions is 37.36%, 28.85% and 33.8%, respectively (**Fig. 40A**). In human endometrium, whereas hypomethylated CpGs we mainly located in promoter regions (67.8% versus 16.0% and 16.2% in intergenic and gene body regions, respectively) (**Fig. 40B**); hypermethylated CpGs were more frequently enriched in gene bodies (53.1% versus 28.2% and 18.7% in intergenic and promoter regions, respectively) (**Fig. 40C**). These distributions largely differed from the genomic CpGs distribution in the MethylationEPIC array (**Fig. 40A**).

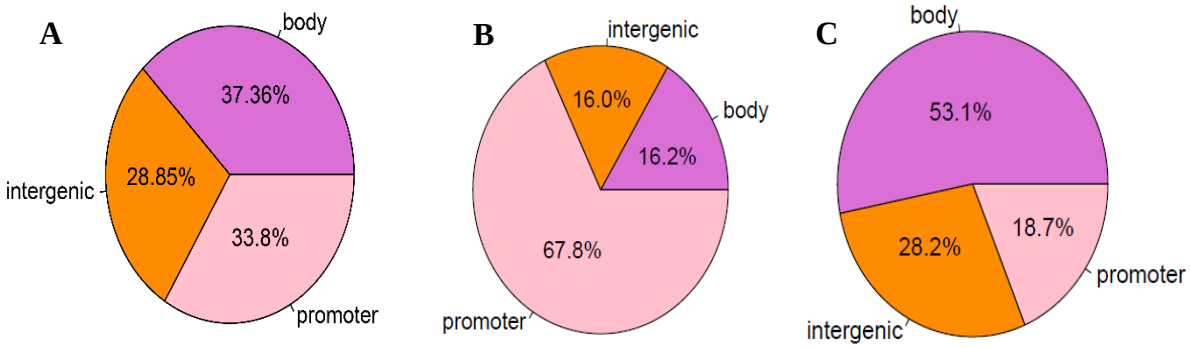


Figure 40. Pie diagrams representing the genomic distribution of CpGs in A) MethylationEPIC BeadChip array or (B-C) human endometrium: B) hypomethylated (β ≤0.2) and C) hypermethylated (β ≥0.8) CpGs.

4.3.2. Comparative analysis of regions with conserved hypo-/hyper- methylation among individuals

Finally, we aimed to get further insights into the endometrial DNA methylome through the identification of loci displaying conserved DNA methylation levels (SD <0.2) in all subjects as well as loci showing different DNA methylation patterns (‘‘variable’’). We analysed the CpG content and neighbourhood context, assuming the following definitions: 1) ‘‘island’’ as a DNA sequence (>200-bp window) with a GC content greater than 50% and an observed: expected CpG ratio of more than 0.6; 2) ‘‘shore’’ as a sequence 0–2 Kb distant from the CGIs; 3) ‘‘shelf’’ as a sequence 2–4 Kb distant from the CGIs; 4) ‘‘open sea/ others’’ as the remaining sequence.

Results were represented in a Mosaic plot, in which the area of each square is proportional to the number of CpGs in each category (**Fig. 41**). We observed the majority of CpGs with conserved levels of methylation among individuals are located in open sea CpG context. Within this group, intermediately methylated CpGs represent the most numerous group. Regarding CGIs, the majority of CpGs were found to be hypomethylated, followed by intermediately methylated CpGs and a reduced group of methylated CpGs.

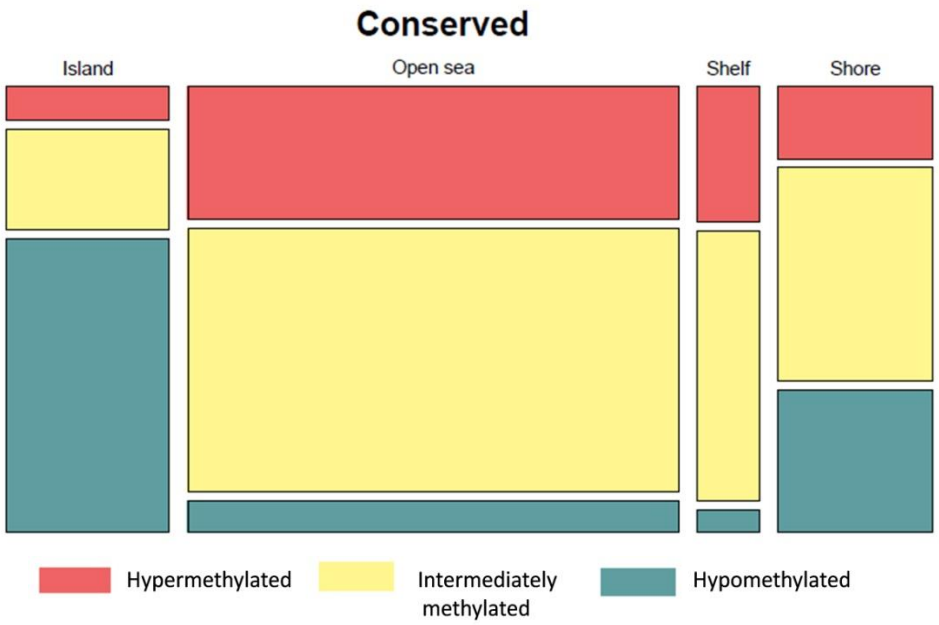


Figure 41. Mosaic plot of CpGs with conserved methylation levels among individuals. Hypomethylated (β ≤0.2); Hypermethylated (β ≥0.8); Intermediately methylated (0.2< β <0.8)

4.4. DNA methylation analysis of selected genes

4.4.1. DNA methylation analysis of the main regulators of angiogenesis

Provided our experience in the study of angiogenesis in endometriosis (Cosín et al, 2010; Ramón et al, 2011; Braza-Boïls et al, 2013, 2014, 2015; Marí-Alexandre et al, 2015), we wanted to analyse the methylation status of the genes of the main regulators of angiogenesis (VEGF-A and TSP-1) in our samples. Thus, we analysed 32 CpGs belonging to *VEGFA*: 23 CpGs localized in the *VEGFA* promoter region and 9 in the gene body (**Fig. 42A**); and also, 51 CpGs belonging to *THBS1*, 24 of which corresponded to the promoter region and 27 to the gene body (**Fig. 42B**). As can be observed, identical methylation levels were found for the CpGs analysed between EUT and CNT. Regarding their genomic localization, CpGs at both the *VEGFA* and *THBS1* promoters were found to be hypomethylated, whereas CpGs at body genes were found to be hypermethylated.

4.4.2. DNA methylation analysis of the main regulators of fibrinolysis

Additionally, we wanted to determine the methylation status of the CpGs related to the urokinase gene (*PLAU*) and the PAI-1 gene (*SERPINE1*). Thus, we analysed 22 CpGs corresponding to *PLAU*, of which 16 CpGs localized in the promoter region and 6 CpGs to the gene body; and 15 CpGs for *SERPINE1*, of which 9 localized in the promoter and 6 in the gene body. In agreement with results for pro- and anti-angiogenic factors analysed, CpGs at promoter regions are hypomethylated, whereas CpGs at body genes remain methylated. Although with a minimal difference for *PLAU*, β-values for all CpGs were found to be overlapping between EUT and CNT endometrium.

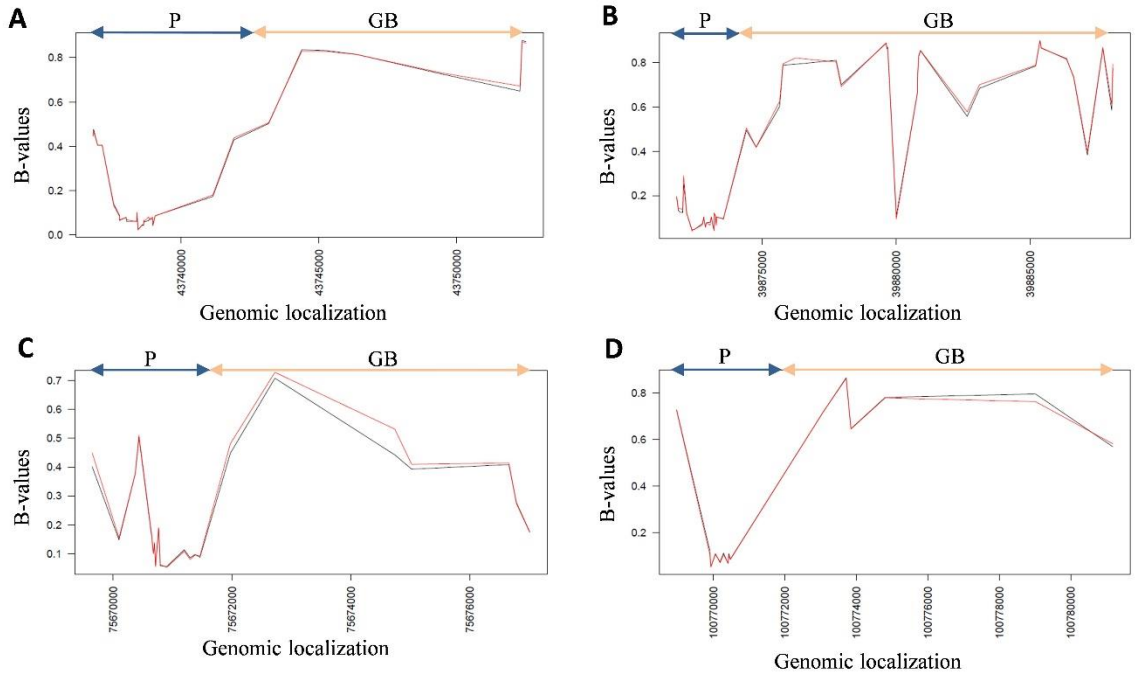


Figure 42. Methylation levels (represented as β-values in y-axis) of the main regulators of angiogenesis and fibrinolysis. A) Vascular endothelial growth factor A, *VEGFA*; B) Thrombospindin-1, *THBS1*; C) urokinase, *PLAU*; D) plasminogen activator inhibitor 1, *SERPINE1*. Genomic localization is represented in the x-axis. The regions corresponding to the promoters and to the gene bodies are highlighted. Red and black lines represent median β-values for patients with endometriosis and control women, respectively. P: Promoter; GB: Gene body.

4.5. Discussion

In this study, we wished to perform DNA methylation profiles in endometrial samples from patients with endometriosis and from control women (paired by age and cycle phase) by employing the Infinium MethylationEPIC BeadChip from Illumina.

The importance of age on DNA methylome has been well established by several authors, who revealed a global loss of DNA methylation and promoter hypermethylation of genes with a dual role in tumour suppression and progeria with aging (Issa et al, 2003; Fraga and Esteller, 2007). Besides, Houshdaran and co-workers (Houshdaran et al, 2014) reported the influence of the phase of the menstrual cycle on DNA methylation patterns in endometria from women without endometriosis. In our study, we did not find significantly differences neither between the age of patients and control women, nor in the proportion of menstrual, proliferative and secretory phases. Thus, we avoided these confounding factors in our results.

We studied endometrial samples from 13 women with endometriosis (stage III-IV) and 11 women without endometriosis, proportionally distributed across the menstrual cycle. Supervised hierarchical cluster was unable to discriminate patients from controls' samples, suggesting that differences, if any, would involve a reduced number of the 866,836 CpGs analysed. This is further supported by the high degree of correlation of the CpGs-methylation status in patients and controls' samples, demonstrated by scatter plots. By means of elastic net regression model, we were able to determine 7 CpGs which together provided the highest discrimination power between patient and control women's samples. Of them, three CpGs had not been assigned to any known gene (namely CpG11496778, CpG01378439 and CpG04296894) and the remaining four CpGs were associated with genes not previously related to endometriosis (namely LDL receptor related protein 5, *LRP5*; Ssemaphorin4F, *SEMA4F;* myosin light chain 12B, *MYL12B;* and phosphoinositide-3-kinase adaptor protein 1, *PIK3AP1*). Nevertheless, differences in methylation status for the 7 CpGs between patients and controls' samples are negligible, regardless of the phase of the menstrual cycle considered.

Apart from papers evaluating DNA methylomes in primary cell cultures from EUT and CNT (Yamagata et al, 2014; Yotova et al, 2017), only three papers analysing the changes in DNA methylation patterns in the endometrial tissues from women with and without endometriosis have been published (Naqvi et al, 2014; Saare et al, 2016; Houshdaran et al, 2016). Several differences in study design could explain the diversity in results among these studies and with respect to ours.

Two of these studies (Naqvi et al, 2014; Houshdaran et al, 2016) were performed with the Illumina Infinium HumanMethylation27 BeadChip assay and the other (Saare et al, 2016) was performed with the Infinium Human methylation 450K BeadChip assay. Therefore, to date our study is the only one employing the new Illumina Infinium HumanMethylationEPIC BeadChip assay for evaluating DNA methylation profiles in EUT and CNT tissues.

Naqvi and co-workers (Naqvi et al, 2014) validated the methylation status of the top 5 hypermethylated genes (namely *MGMT*, *DUSP22*, *CDCA2*, *ID2* and *RBBP7*) and hypomethylated genes (namely *TNFRSF1B*, *BMPR1B*, *ZNF681*, *IGF21* and *TP73*) found in the methylation array comparing endometrial DNA from 7 patients with endometriosis

and 6 endometriosis-free controls. Although pioneer in the field, this study presents several limitations that hinders extrapolating results to other studies. For instance, few data are reported with respect to factors known to modify the methylome (such as age and phases of the menstrual cycle). Additionally, differences are reported as fold-change, rather than with the standard β-values, which imped determining the magnitude of methylation change.

Later on, Saare and co-workers (Saare et al, 2016), from Dr. Peter's laboratory, analysed the major cohort of endometrium from patients with endometriosis (n=31) and control women (n=24) reported to date, by means of the Infinium HumanMethylation450K BeadChip assay. In this case, they took into account the three menstrual phases (additionally classifying secretory phase in early-, middle- and late-secretory) and age, among other considerations. In agreement with our own results, they observed that the DNA methylation profile of patients and controls was highly similar, finding only 28 statistically significant differentially methylated regions (DMRs) (16 of which associated to known genes). Nevertheless, the Δβ for these DMR was rather small (from –0.01 to –0.16 for hypomethylated CpGs and from 0.01 to 0.08 for hypermethylated CpGs), similarly to what we observed for the 7 CpGs determined by the elastic net statistical analysis. Unfortunately, these Δβ are far from the threshold of $\Delta\beta \geq 0.136$, established to differentiate biological relevant methylation changes from background noise and platform variability (Bibikova et al, 2009). Interestingly, when comparing samples by cycle phase (patients and controls together), they observed the largest epigenetic changes occurring during the late-secretory and menstrual phases, when substantial rearrangements of endometrial tissue take place (Saare et al, 2016).

The same year, Houshdaran and co-workers (Houshdaran et al, 2016) published the last paper to date on this field. They analysed 17 patients with endometriosis and 16 control-free women, taking also into account age and the phase of the menstrual cycle, among other considerations. In contrast with our experimental design, they excluded samples in in menstrual phase. As far as we are concerned, possible differences in menstrual phase samples could be of a great interest to develop a non-invasive biomarker for endometriosis, as successfully achieved for the detection of endometrial cancer in DNA isolated from vaginal secretion collected from tampons (Fiegl et al, 2004).

Regarding differences between patients and controls, they found the largest differences in middle secretory samples, followed by proliferative and early secretory samples. However, the overall median β-value differences of CpG sites with biological relevance in endometrium was again rather small (0.173 [0.14, 0.31] for hypermethylated CpGs and -0.162 [-0.31, -0.14] for hypomethylated CpGs) (Houshdaran et al, 2016). These subtle differences could explain why authors did not conduct a bisulphite sequencing validation phase.

Due to the still high cost per sample of DNA methylome analysis, an ideal approach for epigenetic-based biomarkers is to select candidate CpGs to be validated by bisulphite sequencing, establishing these technique as the reference for CpG methylation assessment in a more affordable manner. Nevertheless, taking together the studies from Houshdaran and collaborators (Houshdaran et al, 2016), Saare and collaborators (Saare et al, 2016) and our own data, it seems clear that DNA methylation differences in endometrium from patients with endometriosis and control women are too light to be considered as non-invasive biomarkers in a clinical affordable bisulphite sequencing approach. This adds to

the striking finding that no single CpG is in common among the three aforementioned studies and our own data, what also precludes establishing a candidate CpG as biomarker.

Regarding the characterization of genome-wide endometrial methylome, the density plot representing frequency of CpGs with specific methylation shows a bimodal distribution of CpGs (**Fig. 39**), with 23.3% of them hypomethylated ($\beta \leq 0.2$), whereas 24.8% of the CpGs were hypermethylated ($\beta \geq 0.8$). Additionally, when looking at functional distribution of CpGs, we observed an enrichment of promoters in hypomethylated regions, whereas hypermethylated CpGs were enriched in gene body regions. These results are in agreement with previous published papers (Kukushkina et al, 2016). On the one hand, promoters at genomic regions involved in active transcription are hypomethylated resulting in a relaxed structure of chromatin and accessibility to transcription factors (Lokk et al, 2014). On the other hand, hypermethylation at body genes is positively correlated with gene expression (Hellman and Chess, 2007), what has been proposed to be related to elongation efficiency and prevention of spurious initiations of transcription (Zilberman et al, 2007). On overall, these methylation profiles would guarantee the accessibility of transcription factors in a tissue with important cyclic changes in gene expression under hormonal influence and also gene stability in a tissue with a high mitotic activity as the endometrium.

As far as CpG context is concerned, we found a predominant hypomethylation at CGI, representing the hypermethylated group only a small fraction of CGI, in agreement with other authors (Houshdaran et al, 2016; Kukushkina et al, 2016).

Finally, since it is currently acknowledged that different epigenetic modifications work together to regulate the functioning of the genome (Sharma et al, 2010), we aimed to investigate whether DNA methylation of the main regulators of angiogenesis (i.e. *VEGFA* as activator and *THBS1* as inhibitor) and fibrinolysis (i.e. *PLAU* as activator and *SERPINE1* as inhibitor) could be involved in the up-regulation of their protein levels in endometrial tissue from patients in comparison to endometrial tissue from control women (see Results section, Chapter 1, **Fig. 21A**), in addition to the regulation by miRNAs described in previous chapters (see Chapter 1, Chapter 2 and Chapter 3). In all cases, we observed that CpGs at promoters of the four genes were hypomethylated, whereas CpGs at body genes were mainly hypermethylated. This is in agreement with an active state of transcription for these genes in the endometrium and a notable gene stability, as previously mentioned.

With respect to angiogenesis, the up-regulation of *VEGFA* mRNA expression in the EUT of patients with endometriosis in comparison to the endometrium of women without the disease has been reported by several authors, including our research group (Takehara et al, 2004; Gilabert-Estellés et al, 2007; Ramón et al, 2011). Furthermore, several papers have reported the regulation of *VEGFA* expression by DNA methylation at specific CpGs of the *VEGFA* promoter (Siddique et al, 2013; Ping et al, 2013). The DNA methylation profile of *VEGFA* described herein denotes an active transcriptional state for this gene in the endometrium, paving the way for the action of transcription factors as HIF-1α, oestrogen receptor, NF-κβ (Shibuya et al, 2008) and β-catenin (Zhang et al, 2015).On overall, this suggests a predominant regulation of other factors (e.g. transcription factors, miRNAs) rather than DNA methylation in the differential expression of VEGF-A in the endometrium from women with and without endometriosis, reinforcing the importance of results detailed in Chapter 1, Chapter 2 and Chapter 3. Additionally, if these results

happen to be validated by bisulphite sequencing, this could pave the way to develop epigenetic therapies specifically targeting and methylating the *VEGFA* promoter to decrease their expression in endometrium from patients with endometriosis.

With regards to *THBS1*, *PLAU* and *SERPINE1*, our research group have previously reported increased levels of their transcripts in EUT *vs.* CNT, although without reaching statistical significance (Gilabert-Estellés et al, 2007). However, we have also described that protein levels are significantly increased in EUT *vs.* CNT tissues (Braza-Boïls et al, 2014). This would support the involvement of a post-transcriptional mechanism of regulation; highlighting the importance of miRNA regulation of these proteins partially deciphered in this work (see Chapter 1 and Chapter 3).

Chapter 5

Chapter 5. Epigenetic editing of the *VEGFA* promoter in primary endometrial stromal cell cultures

Several studies, including ours, have reported an increase of the main regulator of angiogenesis, VEGF-A, both at the mRNA (Takehara et al, 2004; Gilabert-Estellés et al, 2007; Ramón et al, 2011) and the protein level (Braza-Boïls et al, 2014) in the EUT in comparison to CNT. Additionally, the regulation of *VEGFA* transcription by CpG methylation at its promoter has also been documented (Siddique et al, 2013; Ping et al, 2013). Since angiogenesis is essential for the survival and implantation of migrated eutopic tissue to the peritoneal cavity and establishment of the endometriotic lesions (Laschke and Menger, 2007; Braza-Boïls et al, 2014; Marí-Alexandre et al, 2015), we aimed to develop a therapeutic tool based on targeted epigenetic editing at the *VEGFA* promoter.

It is noteworthy to mention that the experiments described in this Chapter were performed in the framework of my pre-doctoral stay in the Institutefor Biochemistry of the University of Stuttgart (Germany), under the direction of Prof. Dr. Albert Jeltsch.

5.1. Characteristics of patients included in the study

Characteristics of patients included in the study are summarized in **Table 12**.

In order to perform primary stromal cell cultures, 3 endometrial tissues (eutopic cells) (mean age 36 years; range 30-40) from women with moderate-severe endometriosis (stages III and IV) and 2 endometrial tissues (control cells) from women without the disease (control cells) (mean age 35.5 years; range 34-37) were obtained. Notably, tissues from one patient with endometriosis and from the two control women had also been analysed in Chapter 5.

Table 12. Clinical characteristics of patients included in the study.

	CONTROL WOMEN (n = 2)	**ENDOMETRIOSIS** (n = 3)
AGE (YEARS; MEAN± SEM) **RANGE** (YEARS)	35.5 [34 - 37]	36.0 [30 - 40]
CYCLE PHASE		
• *PROLIFERATIVE*	n = 1 (50.0%)	n = 1 (33.3%)
• *SECRETORY*	n = 1 (50.0%)	n = 2 (66.6%)
• *MENSTRUAL*	n = 0 (0%)	n = 0 (0%)
ENDOMETRIOTIC LESIONS		
• *OMA*	NA	n = 2 (66.6%)
• *PI*	NA	n= 0 (0%)
• *RVN*	NA	n= 1 (33.3%)
STAGE		
• *I-II*	NA	n = 1 (33.3%)
• *III-IV*	NA	n= 2 (66.6%)

NA: not applicable

5.2. Validation of the methylation status at selected CpGs of the *VEGFA* promoter

We initially wished to validate the methylation status at 11 selected CpGs at the *VEGFA* promoter by means of bisulphite sequencing. The genomic context of the selected CpGs is illustrated in **Fig. 43** and additional data is provided as Supplementary material (**Annex 2, Suppl. Table 3**).

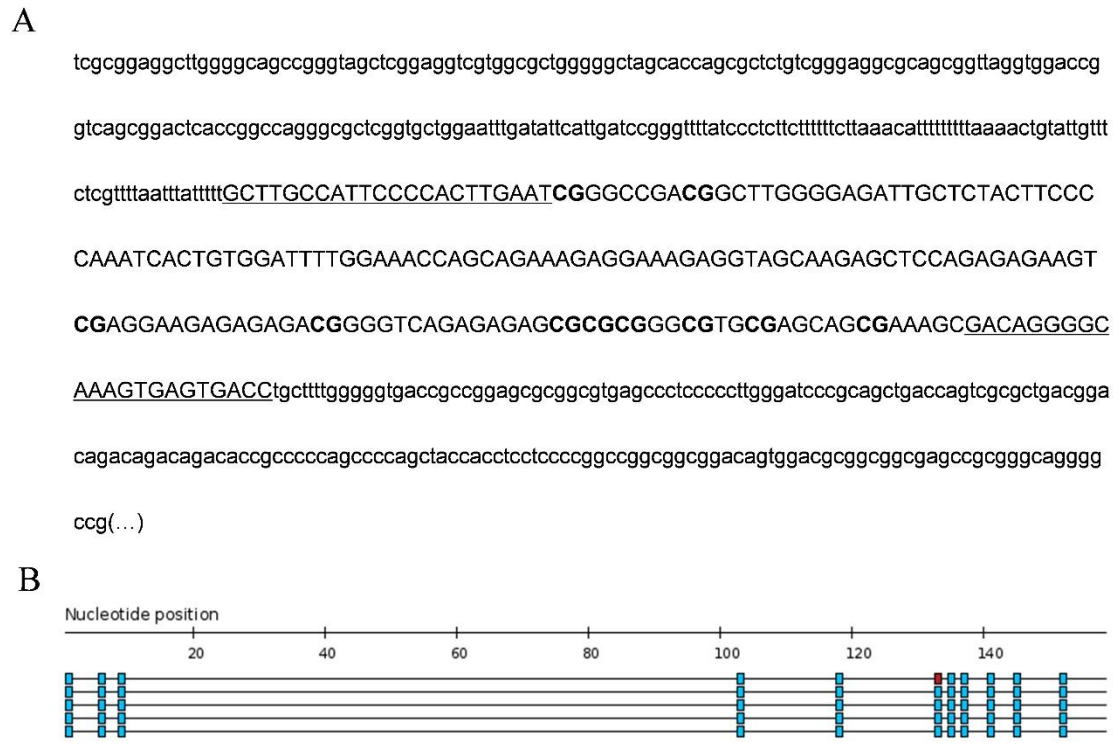


Figure 43. Genomic localization of the 11 CpGs analysed by bisulphite sequencing within the *VEGFA* promoter. A) 600 nt of *VEGFA* promoter, including the 11 CpGS analysed are represented nucleotides from (position: 43738182 – 43738772). Amplicon is in capital letter, sites for primer pairing are underlined and the analysed CpGs are in bold. B) Illustrative example showing the reference CpG sites in context of the sequence length.

Primary stromal eutopic and control endometrial cells were grown to passage 3, and bisulphite sequencing was performed on extracted DNA in untransfected cells (See Materials and Methods section, point 7.2.1. for further details). 5 colonies were evaluated for each primary stromal cell culture of eutopic and control cells (except for one eutopic cell culture, in which only 3 colonies were evaluated). Sequencing results are represented as condensed diagrams, in which each row corresponds to one clone of bisulphite PCR product and each column corresponds to one reference CpG. The colour code indicates different methylation states (blue: unmethylated; red: methylated). Sequencing results showed that the methylation level was 0% in control cells and 1.2±0.17 % in eutopic cells, confirming a hypomethylation status at the *VEGFA* promoter and enabling the delivery of targeted methylation with targeted epigenetic therapies (**Fig. 44**).

Once data from arrays had been validated, we focused on eutopic cells to perform the following experiments.

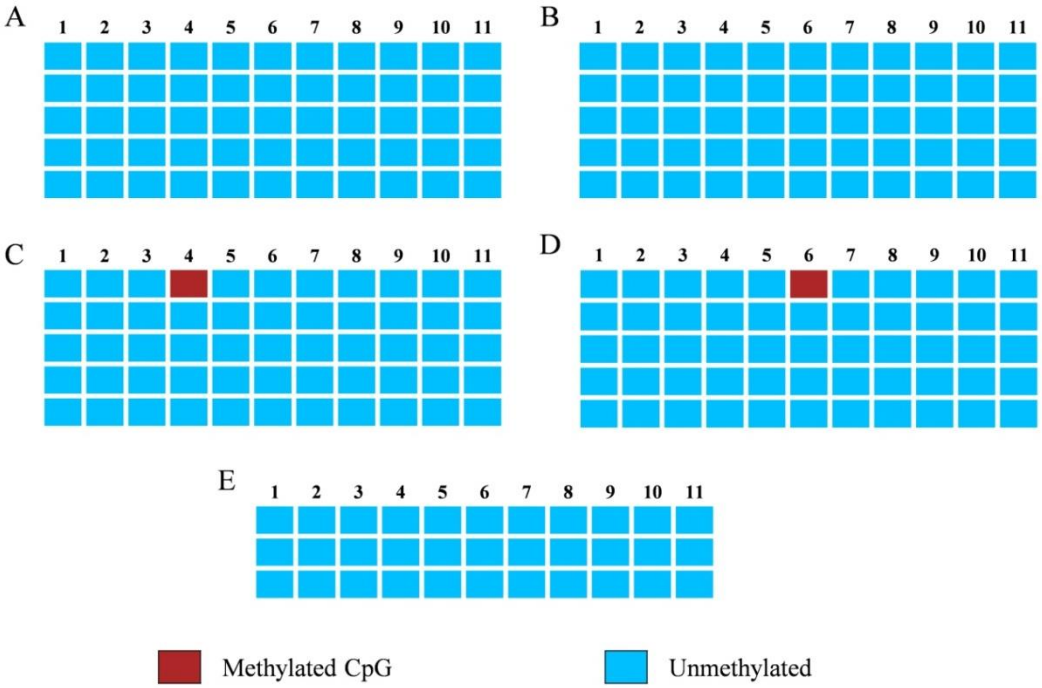


Figure 44. Validation of the methylation status at the *VEGFA* promoter by bisulphite sequencing of DNA from control (A, B) and eutopic cells (C-E). As observed, bisulphite sequencing denoted a global hypomethylated status of the DNA at the *VEGFA* promoter.

5.3. Analysis of *VEGFA* mRNA expression over cell culture passages

We next aimed to evaluate the possible variation of *VEGFA* mRNA levels in untransfected primary stromal endometrial cell cultures over passages. This would enable us to take into account physiological turnover of *VEGFA* mRNA and eliminate a possible source of confounding data. To this end, we isolated total RNA at three different points during cell culture (passage 3, passage 5 and passage 7) and performed qRT-PCR quantification of *VEGFA* mRNA. As can be observed (**Fig. 45**), *VEGFA* mRNA expression decreases over passages, with a drastic reduction in later passages (16.7% and 81.3% of reduction in passages 5 and 7 in comparison to passage 3, respectively). Therefore, we established passage 3 as the optimal for transfection experiments.

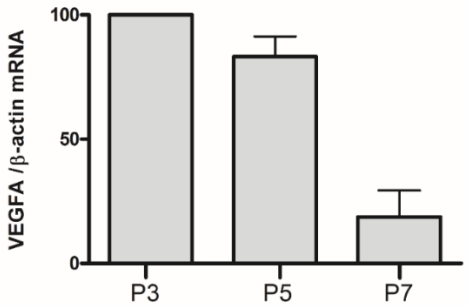


Figure 45. qRT-PCR quantification of *VEGFA* mRNA expression in primary untransfected eutopic stromal cells over passages. Data are expressed as percentages relative to passage 3. Percentages are the average of three independent experiments and error bars correspond to the standard error of the mean. P3: passage 3, P5: passage 5; P7: passage 7.

5.4. Targeted methylation delivery at the *VEGFA* promoter of primary eutopic stromal cells

To deliver specific methylation at the *VEGFA* promoter of our primary stromal cells we employed an optimized fusion protein, previously employed at the Prof. Dr. Jeltsch's laboratory (Siddique et al, 2013). This fusion protein encodes the C-terminal domain of the murine Dnmt3a (Dnmt3a-C), the C-terminal domain of the human Dnmt3L and an artificial zinc-finger protein targeting the *VEGFA* promoter. For simplicity purposes, this fusion gene would be defined as VAZF3a3L from now on.

5.4.1. Transfections employing the FuGene® HD reagent (Promega)

Following previous successful protocols employed at Prof. Dr. Jeltsch's laboratory, we transfected primary eutopic stromal cells with plasmids for VAZF3a3L and GFP (as reporter gene), employing the FuGene® HD transfection reagent (Promega). Additionally, HEK-293 cells (ATCC) served as a positive control for transfection. Confocal microscopy analyses were scheduled every 24h, up to a final time of 72h.

Results showed a low transfection efficiency in primary eutopic stromal cells at 72h, although cells maintained their characteristic spindle-shaped fibroblastic morphology (**Fig. 46A-C)**. This would suggest a low toxicity for the reagent employed. Oppositely, the same experimental settings allowed a time-dependent increase of the percentage of transfected (GFP+) HEK-293 cells (**Annex 3, Suppl. Fig. 3**) with a maximal transfection efficiency at 72h (**Fig. 46**). These results spurred us to try a new strategy for a successful transfection.

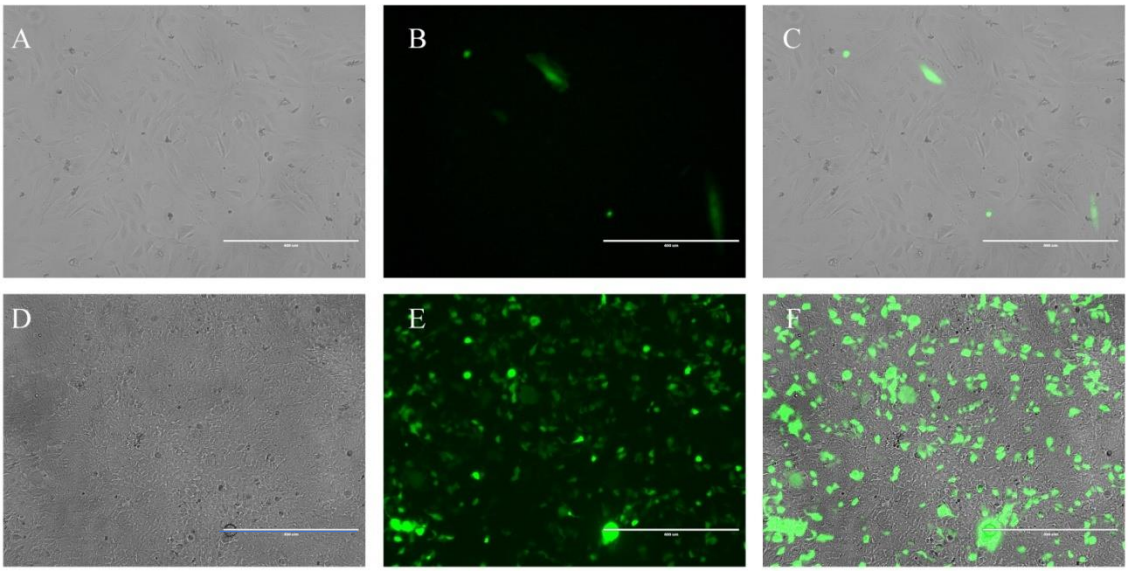


Figure 46. Analysis of transfection efficiency after 72h of FuGene® HD transfection in HEK-293 cells (A-C) and primary eutopic stromal cells (D-E). A) and D) Optical microscopy for morphological analysis. B) and E) Confocal microscopy for green fluorescent reporter protein analysis of transfection efficiency. C) and F) Merged image. (Scale 1:45).

5.4.2. Transfections employing Lipofectamine™3000 reagent

With the aim of increasing the efficiency of transfection, we moved to another transfection reagent, Lipofectamine™ 3000 (Thermofisher Scientific), which is an updated formula of the widely employed Lipofectamine™ 2000.

a. Reagent titration

In order to optimize the experimental conditions a dose-dependent experiment was performed using two Lipofectamine doses. Cells were seeded in 6-well plates at a density of $2 \cdot 10^5$ cells/well. Transfections were performed after 24h, when cells reached 80% of confluency. To this, we used plasmids for VAZF3a3L and GFP, at two different doses of Lipofectamine™ 3000: 3.75 µL and 7.5 µL.

Transfection efficiency was again teste by fluorescent microscopy to assess the expression of GFP. As observed (**Fig. 47**), the higher dose of the reagent (7.5 µL) exerts a more deleterious effect on primary eutopic endometrial stromal cells, without improving the percentage of GFP(+) cells (**Fig. 47A**). Cytometric analyses showed a moderate efficiency of transfection in both conditions (in the example, 31,11% *vs*. 29, 32 for 3.75 µL and 7.5 µL, respectively) (**Fig. 47B**). Therefore, subsequent experiments were performed with 3.75 µL of Lipofectamine™ 3000.

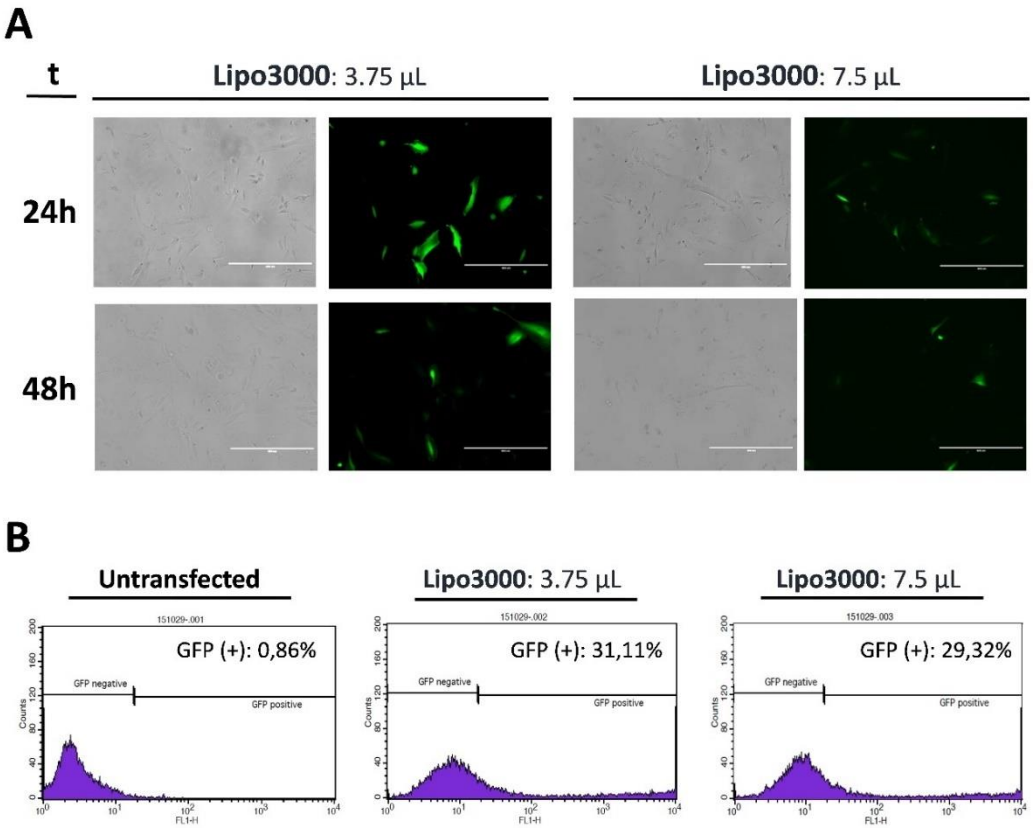


Figure 47. Optimization of the Lipofectamine™ 3000 dose for eutopic stromal cells transfections. A) For each volume of the reagent employed, (3.75 µL and 7.5 µL) optical microscopy (left columns) and fluorescent microscopy (right column) images were obtained at two time-points: 24 h, upper row; and 48 h, lower row (Scale 1:25). B) Cytometric analyses of transfection efficiency (GFP(+) cells). Left image represents control untransfected cells. Lipo3000: Lipofectamine™ 3000 reagent (Promega). GFP: Green fluorescent protein.

b. Analysis of transfection efficiency after Magnetic activated cell sorting enrichment

Provided the nature of transient transfections, determinations wouldhave to be done in a mixed population of transfected and untransfected cells. Thus, we next wished to increase the percentage of transfected cells, since an overpopulation of untransfected cells could mask the phenotype achieved in successfully transfected endometrial stromal cells.

To this, we co-transfected the plasmids for VAZF3a3L and GFP with an additional plasmid encoding atruncated human low-affinity nerve growth factor receptor (LNGFR; Miltenyi Biotech). This latter plasmid expresses a truncated membrane surface receptor that is targeted by a specific antibody labelled to a magnetic bead (MACSelect Microbead; Miltenyi Biotech). Under the effect of a magnetic field, labelled cells are retained, and once eluted constitute the enriched fraction. Oppositely, unlabelled cells are not retained within the magnetic field and are discarded (flow-through fraction).

Afterwards, we monitored transfection efficiency every 24 h in a two time-points schedule (**Fig. 48A**), when cells were harvested to proceed with cytometric analyses (**Fig. 48B**). As observed, the transfection efficiency increased around 2.5-times, although even after MACSelect enrichment the transfection yields did not reach the 60% and 80% of efficiency obtained in other cell lines (Siddique et al, 2013; Kungulovski et al, 2015). Remarkably, GFP(+) cells were also obtained in the flow-through fraction, pointing to a different transfection performance of the plasmids employed.

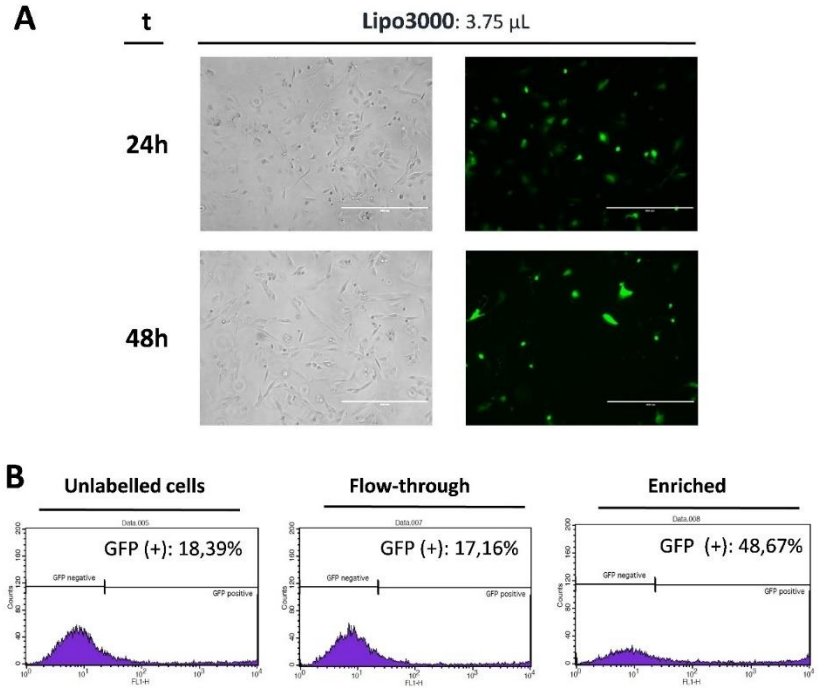


Figure 48. Analysis of transfection efficiency after MACSelect. A) Optical microscopy (left column) and fluorescence (right column) images at two time-points: 24 h and 48 h post-transfection (Scale 1:25). B) Cytometric analyses of cells without MACSelect Microbead labelling (left), unretained fraction (flow-through, middle column) an enriched fraction (right column). Lipo3000: Lipofectamine™ 3000 reagent (Promega). GFP: Green fluorescent protein.

5.4.3. Transfections employing Lipofectamine™ LTX reagent

In order to overcome the aforementioned limitations in terms of efficiency, we decided to transfect cells with Lipofectamine™ LTX reagent (ThermoFisher Scientific), since we had previously been successful with this reagent in functional miRNA studies on primary eutopic stromal cells (Chapter 3).

a. Reagent titration

To optimize the dose for the subsequent experiments, we first performed transfections with plasmids for VAZF3a3L and GFP and increasing concentrations of Lipofectamine™ LTX reagent (5 μL, 7.5 μL and 10 μL) (Thermofisher Scientific) (**Fig. 49**). Analysis of transfection yield determined by cytometry revealed a dose-dependent efficiency of transfection, with the maximum efficiency obtained with 10 μL of the reagent. Notably, these values were higher than the yields obtained with previous conditions and suited the standards necessary for subsequent analyses (Siddique et al, 2013; Kungulovski et al, 2015). Provided the subtle difference in transfection efficiency between the two upper concentrations of the reagent (73.59% and 72.36% for 10 μL and 7.5 μL, respectively), we decided to employ 7.5 μL for the following experiments, in an attempt to reduce the toxicity of the otherwise quite cytotoxic agent.

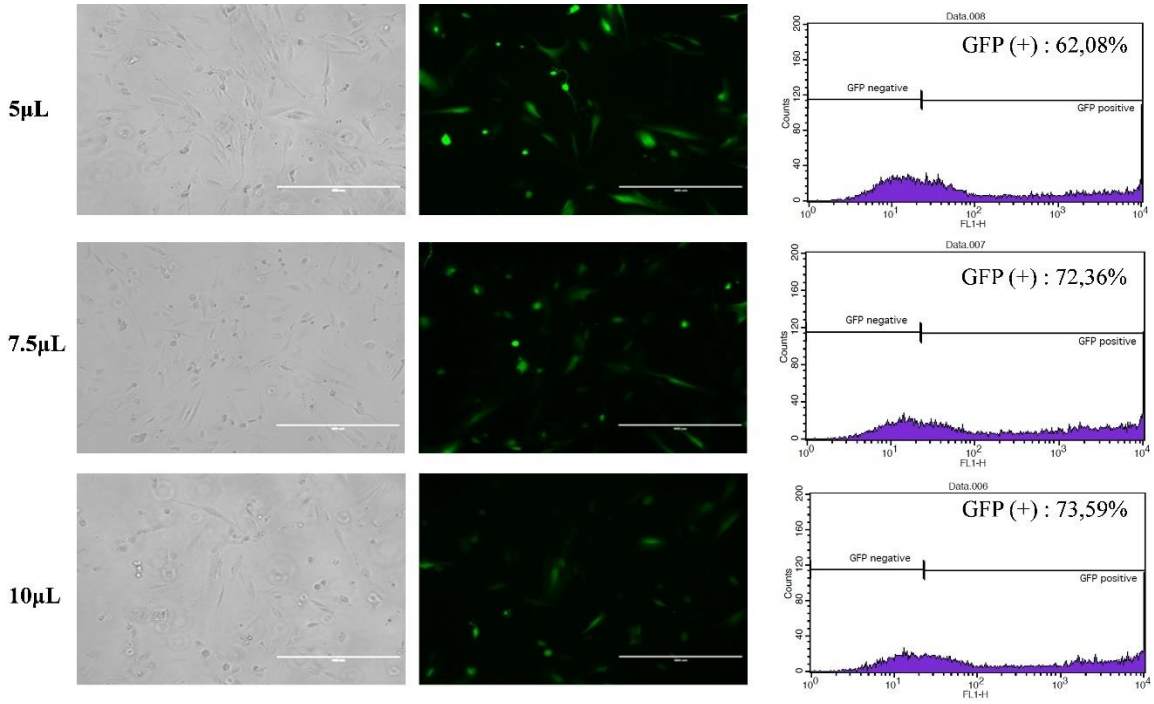


Figure 49. Representative experiment of Lipofectamine™ LTX reagent titration (24h post-transfection). Each row represents a Lipofectamine™ LTX dose. Left column: optical microscopy images. Middle column: confocal microscopy images of GFP (+) cells. Right column: Histogram of GFP(+) cells after transfection, determined by cytometry. The cell count is shown in the vertical axis, GFP fluorescence is given in log scale on the horizontal axis. GFP: Green fluorescent protein.

b. Monitoring of the transfection efficiency over 120h

Previous successful epigenetic editing achieved at Prof. Dr. Jeltsch's laboratory determined an interval of 120 h post-transfection as the optimal time for epigenetic editing evaluation (Kungulovski et al, 2015). Encouraged by the positive results obtained, we decided to up-scale the amount of starting material (from $2 \cdot 10^5$ cells in a 6-well plate to a $2 \cdot 10^6$ cells in a T25 flask) and monitor cell transfection efficiency every 24h until 120h. The increase of starting material was mandatory since multiple aliquots need to be done for cytometric analysis and nucleic acids (RNA and DNA) extractions. Additionally, and aiming to discard the possible toxic effect of the VAZF3a3L insert (2,046 nt), we transfected an empty vector (Adtrack) as a negative control. Results are presented in **Fig. 50**.

At 24 h, few cells expressed GFP and the majority of them were round-shaped, indicating an apoptotic state. This trend was even more clearly appreciate at 48 h. Interestingly, optic microscopy images show an increase in cell population from 72 h to 120 h, although GFP(+) cells decreased to zero during this period of time. This would suggest that untransfected cells but not GFP(+) cells survived and proliferated to repopulate the recipient. Comparing the two experimental conditions, a subtle increase in transfection efficiency was observed in VAZF3a3L(-) empty vector *vs.* VAZF3a3L(+), although cells displayed the same behaviour in both cases in terms of apoptosis, survival and proliferation. This could suggest that the main toxicity was exerted by the reagent, rather than by the length of the VAZF3a3L plasmid.

5.5 Discussion

Exceptionally, in the human endometrium, the process of angiogenesis occurs in physiological conditions, since the functional layer has to be cyclically regenerated from the basal layer after each menstruation (Augustin, 2000). Nevertheless, we and other authors have documented an imbalance towards a more active angionenic state in the EUT from patients with endometriosis (Shiffren et al, 1996; McLaren et al, 2000; Gilabert-Estellés et al, 2007; Ramón et al, 2011; Braza-Boïls et al, 2014) and suggested that this modulation could facilitate the survival of migrated tissues and the establishment of endometriotic lesions (Gilabert-Estellés et al, 2012; Marí-Alexandre et al, 2015). At the molecular level, angiogenesis is tightly regulated by a balance between pro-angiogenic factors and inhibitors (Lawler and Lawler, 2012). Whereas VEGF-A has been postulated as the main activator of angiogenesis (Shibuya et al, 2008), TSP-1 is one of the main repressors (Lawler and Lawler, 2012).

In previous studies, we have observed an overexpression of VEGF-A in EUT from patients, without significant differences in the expression of TSP-1 (Ramón et al, 2011; Braza-Boïls et al 2014). Therefore, we focused on *VEGFA* promoter as a target gene to introduce targeted methylation, since the angiogenic imbalance seems to be due to an overexpression of activators rather than a downregulation of inhibitors.

The experimental design undertaken in this project has considered a possible translation into the clinical practice of the potential results. In agreement with this, the choice of epigenetic editing presents several advantages in front of other techniques for downregulating gene expression. On one hand and compared with miRNAs, the

probability of off-target effects is reduced, since it is well known that a single miRNA can modulate the expression of multiple genes.

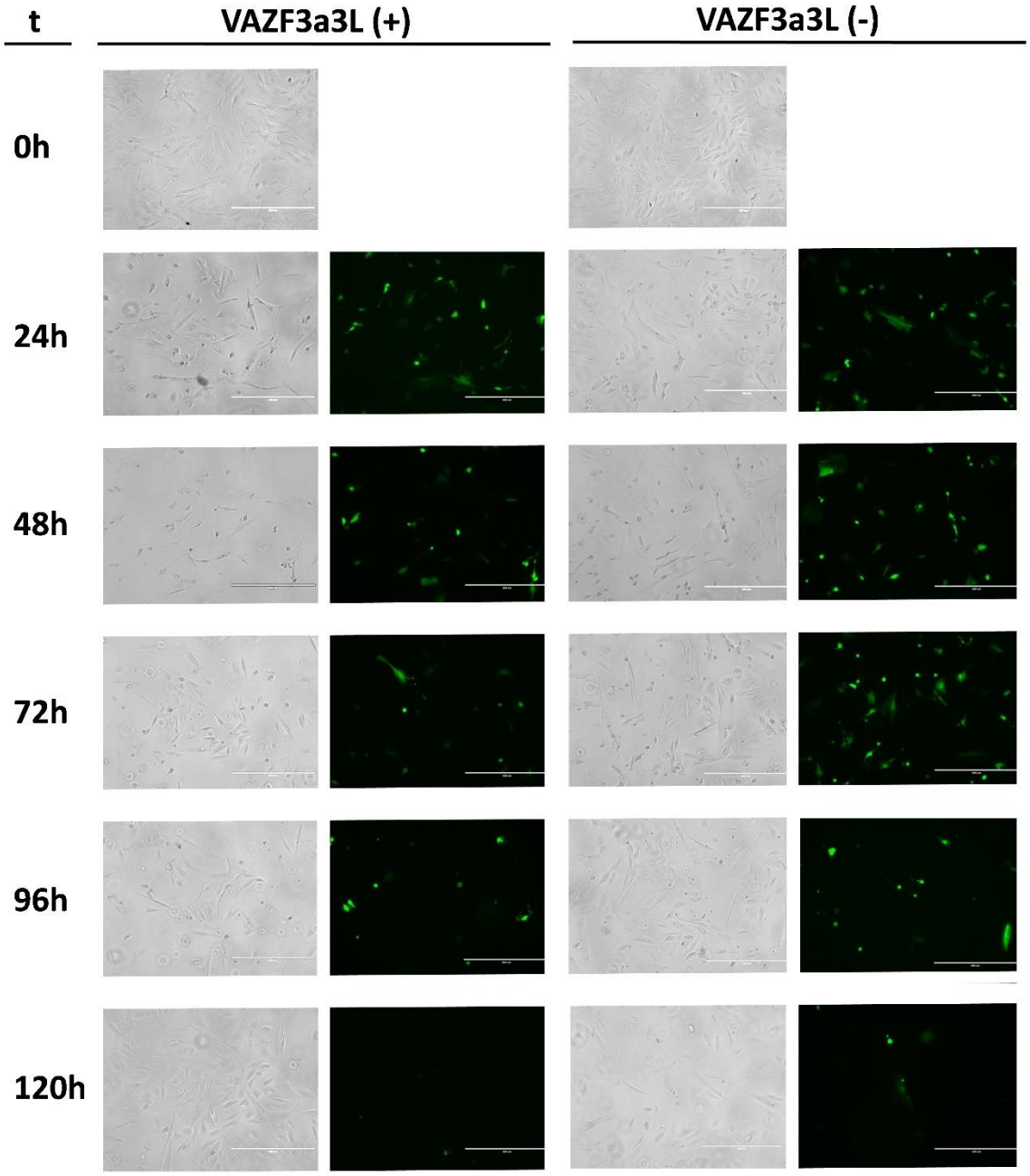


Figure 50. Monitoring cell transfection in two experimental conditions (VAZF3a3L(+) or with an empty vector, VAZF3a3L(-)) each 24h up to a total of 120 h. These experiments were performed in triplicate, obtaining similar results. Each row represents a time point. For each experimental condition, left column display optical microscopy pictures and right column the fluorescence of GFP(+) transfected cells.

Additionally, DNA methylation is to a certain extent conserved through cell divisions (Kungulovski et al, 2015) and cannot be secreted into microvesicles (e.g. exosomes) such as miRNAs (Villaroya-Bertri et al, 2013). On the other hand, and opposite to genetic editing (CRISPR-Cas9 system), this approach would downregulate but not suppress the expression of VEGF-A, in agreement with the published literature for other genes and cell

types (Snowden et al, 2002; Sidiqque et al, 2013; Nunna et al, 2014; Kugunlovski et al, 2015; Stepper et al, 2017). This is crucial, since the endometrium needs to retain a physiological angiogenic capacity to develop important functions, as cyclical regeneration and embryo implantation (Karizbodagh et al, 2017).

In this Thesis, we first we evaluated the methylation status of the *VEGFA* promoter of untransfected eutopic and control endometrial stromal cells. This was performed on 11 CpGs, which had been selected for epigenetic editing due to previous successful reports (Siddique et al, 2013). Bisulphite sequencing results (**Fig. 45**) showed that *VEGFA* promoters were hypomethylated in both eutopic and control stromal cells. This is in agreement with previous results discussed in this Thesis (Chapter 4), showing hypomethylated status of *VEGFA* promoter in EUT and CNT tissues. Provided that hypomethylation at CpG islands of gene promoters is associated with gene expression (Portela and Esteller, 2010; Sandoval et al, 2012), these results denotates an active transcription state for this gene in the human endometrium and highlights the importance of other players, such as miRNAs, in the regulation of VEGF-A (as discussed in Chapter 1, Chapter 2 and Chapter 3). Importantly, the absence of methylation marks at selected CpGs could pave the way for the delivery of targeted methylation.

In order to observe the effect of epigenetic editing on the stromal cells, several steps need to be underwent. Firstly, cells are required to incorporate a plasmid encoding the methyltransferase enzyme and a zinc finger protein conferring locus specificity. Afterwards, cells are to express the encoded protein, reach the nucleus and deposit methylation marks at cytosines of interest and, finally, it is necessary to exceed the half-life time to observe a decrease in the pool of endogenous *VEGFA* mRNA (see Material and Methods section, **Fig. 18**).

In our design, the methyltransferase activity was achieved with a fusion protein containing the C-terminal catalytic domain of the murine Dnmt3 and the C-terminal domain of the human Dnmt3L. Albeit Dnmt3L (Dnmt3-like) does not show intrinsic cytosine methyltransferase activity, due to the mutation of key catalytic residues, it forms a complex with Dnmt3a, increasing about 10-fold times the Dnmt3a DNA methylation activities (Hata et al, 2002). Additionally, our construct also included a region encoding for a multimodular zinc finger protein, with specificity for the *VEGFA* promoter locus. This construct had been previous and successfully employed at the laboratory where experiments were performed (Siddique et al, 2013).

To transfect primary stromal endometrial cells, 3 different reagentswere employed. The first reagent, FuGene® HD transfection reagent (Promega), reproduced the conditions of the aforementioned study (Siddique et al, 2013). Although with low toxicity, few cells were transfected, with prompted us to undertake a different strategy. Then, we used the reagent Lipofetamine™ 3000 (Thermofisher Scientific), which yielded a modest transfection efficiency (**Fig. 47B**). As long as we employed transient transfections, bisulphite sequencing and *VEGFA* mRNA qRT-PCR determinations are to be performed in a population containing both transfected and untransfected cells. For this reason, it is necessary to perform analyses in a population enriched in transfected cells, to avoid masking the results. To this end, we sought to increase the efficiency through magnetic activated cell sorting, which is otherwise a common procedure. Even so, the transfection yields did not reach the minimum required (Siddique et al, 2013; Kungulovski et al, 2015) (**Fig. 48B**).

Finally, we turned to the reagent successfully employed by our group to transfect the same cell types (Braza-Boïls et al, 2015 and Chapter 3). The use of Lipofetamine™ LTX (Thermofisher Scientific) had not been our first choice for two main reasons: 1) we were working with primary cell cultures, far less resistant to cytoxic agents than other cell types, as cancer cell lines and 2) the manufacturer reports a higher toxicity for this agent in comparison to Lipofectamine™ 3000. With this reagent, we notably improved the efficiency of transfection in the experiment performed in a 6-well plate (**Fig. 49**). Encouraged by this, we up-scaled the amount of starting material in order to obtain enough transfected cells to perform all the required experiments (DNA bisulphite sequencing, *VEGFA* mRNA qRT-PCR and cytometric analysis of GFP(+) cells). Additionally, we kept into culture transfected cells up to 120h. This time point post-transfection had been reported to be the time of maximum targeted DNA methylation delivery, as optimized in previous papers (Kungulovski et al, 2015). Nevertheless, the amount GFP(+) cells decreased to zero at 120h (**Fig. 50**), impeding subsequent analyses. Instead, culture flasks were repopulated by untransfected cells.

At the light of our own experience, it is not surprising that all the existing literature on epigenetic editing has been performed either in easily transfectable HEK-293 cells (Vojta et al, 2016; Stepper et al, 2017) or cancer cell lines (Rivenbark et al, 2012 in SUM159 and MCF7 breast cancer cells; Siddique et al, 2013; Nunna et al, 2014; Stepper et al 2017 in SKOV3 ovarian cancer cells, Falahi et al, 2013 in breast cancer cell lines SKBR3, MDA-MB231 and MCF7, in SKOV3 ovarian cancer cell line SKOV3 and in HEK293T cells; Choudhury et al, 2016 in HeLA and MCF7 cancer cell lines and Xu et al, 2016 in the SH-SY5Y human neuroblastoma cell line and HeLA cells) (Reviewed in Marí-Alexandre et al, 2017a). However, no single report exists on primary cell lines.

In conclusion, the absence of methylation marks at the *VEGFA* promoter of eutopic cell lines allows the employment of targeted epigenetic editing to downregulate the expression of this pro-angiogenic factor. Nevertheless, neither the plasmid required is easily transfectable in this primary cell lines, nor they resist the experimental conditions to achieve a successful targeted methylation. In the future, these limitations could be overcome with the employment of adenoviruses and/or the CRIPSR/dCas9 system, although this had been far from the scope of the present project.

V. Final conclusions

V. Final conclusions

Once the hypotheses have been contrasted with the results obtained after developing the proposed objectives, the main conclusions of this Doctoral Thesis are:

1. Both peritoneal and endometrial factors contribute to the pathophysiology of endometriosis.

2. At the tissue level, 157 miRNAs were found significantly differentially expressed (79 up-regulated and 78 down-regulated) in ovarian endometrioma and/or eutopic endometrium in comparison to endometrial tissue from control women.

3. Validation by qRT-PCR of the selected miRNAs (namely miR-29c-3p, -138-5p, -202-3p, -411-5p, -424-5p, -449b-3p and -556-3p) and their correlation with the protein levels of the main regulators of angiogenesis (VEGF-A and TSP-1) suggests the implication of a miRNA-mediated regulation in the establishment and survival of endometriotic lesions.

4. At the peritoneal level, our *in vitro* model has demonstrated to be suitable to study the angiogenic and proteolytic activity involved in the pathophysiology of endometriosis, as well as their regulatory miRNAs.

5. Peritoneal fluid from patients with endometriosis modified the expression of 82 mature miRNAs (72 down-regulated and 10 up-regulated) in primary stromal cell cultures from eutopic endometrium from patients. In the validation phase, 8 selected miRNAs (namely miR-16-5p, -21-5p, -29c-3p, -106b-5p, -130a-5p, -185-5p, -195-5p and -424-5p) showed diminished levels when eutopic cell cultures were treated with peritoneal fluid from patients.

6. We confirmed that *VEGFA* mRNA is as a target of miR-29c-3p, as demonstrated by luciferase assays.

7. There do not exist significant differences throughout the genome-wide methylome between the eutopic endometria from women with endometriosis and the control endometria from women without the disease. The characteristic pattern of DNA methylation of the human endometrium suggests an active transcriptional status in this tissue and highlights the importance of hormonal and miRNA regulation for the modulation of gene expression in this tissue.

8. The absence of methylation marks at the *VEGFA* promoter of eutopic cell lines potentially permits the employment of targeted epigenetic editing to down-regulate the expression of this pro-angiogenic factor. Nevertheless, neither the plasmid required is easily transfectable in this primary cell lines, nor they resist the experimental conditions to achieve a successful targeted methylation.

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VI. References

VI. References

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VII. Annex

VII. Annex

Annex 1: Published papers in which the PhD student has contributed

1. Papers directly related to the Doctoral Thesis
    a. Original research works
    b. Reviews

2. Papers not directly related to the Doctoral Thesis

Annex 2: Supplementary Tables

Annex 3: Supplementary Figures

Annex 1

Annex 1: Published papers in which the PhD student has contributed

1. Papers directly related to the doctoral thesis:

a. Original research works:

1. Braza-Boïls A, Marí-Alexandre J, Gilabert J, Sánchez-Izquierdo D, España F, Estellés A, Gilabert-Estellés J. MicroRNA expression profile in endometriosis: its relation to angiogenesis and fibrinolytic factors. Hum Reprod 2014; 29:978-988.

2. Braza-Boïls A, Gilabert-Estellés J, Ramón LA, Gilabert J, Marí-Alexandre J, Chirivella M, España F, Estellés A. Peritoneal fluid reduces angiogenesis related microRNA expression in cell cultures of endometrial and endometriotic tissues from women with endometriosis. PLoS One 2013; 8:e62370.

3. Braza-Boïls A, Salloum-Asfar S, Marí-Alexandre J, Arroyo AB, González-Conejero R, Barceló-Molina M, García-Oms J, Vicente V, Estellés A, Gilabert-Estellés J, Martínez C. Peritoneal fluid modifies the microRNA expression profile in endometrial and endometriotic cells from women with endometriosis. Hum Reprod 2015. 30:2292–2302.

b. Review papers:

4. Marí-Alexandre J, Diaz-Lagares A, Villalba M, Juan O, Crujeiras AB, Calvo A, Sandoval J. Translating cancer epigenomics into the clinic: focus on lung cancer. Transl Res 2017; 189:76-92.

5. Marí-Alexandre J, Sánchez-Izquierdo D, Gilabert-Estellés J, Barceló-Molina M, Braza-Boïls A, Sandoval J. miRNAs Regulation and Its Role as Biomarkers in Endometriosis. Int J Mol Sci. 2016;17. pii: E93.

6. Marí-Alexandre J, Barceló-Molina M, Olcina-Guillem M, García-Oms J, Braza-Boïls A, Gilabert-Estellés J. MicroRNAs: New players in endometriosis. World J Obstet Gynecol 2016; 5:28-38.

7. Marí-Alexandre J, García-Oms J, Barceló-Molina M, Gilabert-Aguilar J, Estellés A, Braza-Boíls A, Gilabert-Estellés J. MicroRNAs and angiogenesis in endometriosis. Thromb Res 2015;135 Suppl 1: S38-40.

1. Papers directly related to the Doctoral Thesis:

a. Original research works

Human Reproduction, Vol.29, No.5 pp. 978–988, 2014
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human reproduction

ORIGINAL ARTICLE ***Gynaecology***

MicroRNA expression profile in endometriosis: its relation to angiogenesis and fibrinolytic factors

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STUDY QUESTION: Could an aberrant microRNA (miRNA) expression profile be responsible for the changes in the angiogenic and fibrinolytic states observed in endometriotic lesions?

SUMMARY ANSWER: This study revealed characteristic miRNA expression profiles associated with endometriosis in endometrial tissue and endometriotic lesions from the same patient and their correlation with the most important angiogenic and fibrinolytic factors.

WHAT IS ALREADY KNOWN?: An important role for dysregulated miRNA expression in the pathogenesis of endometriosis is well documented. However, to the best of our knowledge, there are no reports of the relationship between angiogenic and fibrinolytic factors and miRNAs when endometrial tissue and different types of endometriotic lesions from the same patient are compared.

STUDY DESIGN, SIZE, DURATION: Case–control study that involved 51 women with endometriosis and 32 women without the disease (controls).

PARTICIPANTS/MATERIALS, SETTING, METHODS: The miRNA expression profiles were determined using the GeneChip miRNA 2.0 Affymetrix array platform, and the results were analysed using Partek Genomic Suite software. To validate the obtained results, 12 miRNAs differentially expressed were quantified by using miRCURY LNA™ Universal RT microRNA PCR. Levels of vascular endothelial growth factor (VEGF-A), thrombospondin-1 (TSP-1), urokinase plasminogen activator (uPA) and plasminogen activator inhibitor-1 (PAI-1) proteins were quantified by ELISA.

MAIN RESULTS AND THE ROLE OF CHANCE: Patient endometrial tissue showed significantly lower levels of miR-202-3p, miR-424-5p, miR-449b-3p and miR-556-3p, and higher levels of VEGF-A and uPA than healthy (control) endometrium. However, tissue affected by ovarian endometrioma showed significantly lower expression of miR-449b-3p than endometrium from both controls and patients, and higher levels of PAI-1 and the angiogenic inhibitor TSP-1. A significant inverse correlation between miR-424-5p and VEGF-A protein levels was observed in patient endometrium, and an inverse correlation between miR-449b-3p and TSP-1 protein levels was observed in ovarian endometrioma. Peritoneal implants had significantly higher levels of VEGF-A than ovarian endometrioma samples.

LIMITATIONS, REASONS FOR CAUTION: Functional studies are needed to confirm the specific targets of the miRNAs differently expressed.

WIDER IMPLICATIONS OF THE FINDINGS: Differences in miRNA levels could modulate the expression of VEGF-A and TSP-1, which may play an important role in the pathogenesis of endometriosis. The higher angiogenic and proteolytic activities observed in eutopic endometrium from patients might facilitate the implantation of endometrial cells at ectopic sites.

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Key words: microRNA / endometriosis / angiogenesis / VEGF-A / fibrinolysis

Introduction

Endometriosis, which is characterized by the presence of endometrial tissue (including glands and stroma) in extra-uterine locations, is one of the most common benign gynaecological diseases (Balasch *et al.*, 1996; Giudice and Kao, 2004; Burney and Giudice, 2012). Like tumour metastases, endometriotic implants require neovascularization if they are to proliferate, invade the extracellular matrix and establish an endometriotic lesion (Laschke and Menger, 2007; Hapangama *et al.*, 2012; McKinnon *et al.*, 2012). There are three clinically distinct endometriotic lesions, which are defined according to their site. Whereas peritoneal implants occur on the peritoneum, endometriomas are cystic lesions sited in the ovary surrounded by endometrioid glands and rectovaginal nodules comprise fibrotic and endometriotic tissue that grow in the rectovaginal space (Gilabert-Estelles *et al.*, 2012; Young *et al.*, 2013). Although it is not clear, whether these endometriotic lesions are variants of the same disease or are different entities (Nisolle and Donnez, 1997; Bulun, 2009), the observed clinical differences require an individualized study of different biological behaviours of each type of lesion.

Although endometriosis is a widely studied disease with a significant impact on the quality of life of patients, the complete pathogenic mechanism remains unresolved. It is believed to be a multifactorial and polygenic disease that involves angiogenesis and proteolysis (Shifren *et al.*, 1996; Donnez *et al.*, 1998; McLaren, 2000; Tan *et al.*, 2002; Gilabert-Estellés *et al.*, 2006, 2007, 2012; Cosín *et al.*, 2009). Our group has reported increased levels of angiogenic and proteolytic factors in endometrium from patients with endometriosis (Gilabert-Estellés *et al.*, 2003, 2005, 2007; Ramón *et al.*, 2005; Cosín *et al.*, 2009, 2010), and indicated that this situation might facilitate the invasive potential of endometrial cells.

Emerging data suggest that dysregulation of microRNA (miRNA) expression may be implicated in the development of endometriosis (Pan *et al.*, 2007, Pan and Chegini, 2008; Toloubeydokhti *et al.*, 2008; Burney *et al.*, 2009; Guo, 2009; Ohlsson-Teague *et al.*, 2009, 2010; Hawkins *et al.*, 2011; Gilabert-Estellés *et al.*, 2012; Braza-Boïls *et al.*, 2013; Laudanski *et al.*, 2013). The apparent abilities of miRNAs to potently regulate the changes in gene expression responsible for endometriosis raise the prospect of using miRNAs as biomarkers and/or therapeutic tools for the disease.

miRNAs are small non-coding RNAs that bind to target mRNAs through translational repression and/or mRNA degradation (Bartel, 2004, 2009). Functional analysis of miRNAs has indicated their regulatory influence on the expression of target genes involved in both physiological and pathological conditions (Ambros, 2004; Burney *et al.*, 2009; Ohlsson-Teague *et al.*, 2009, 2010; Kuokkanen *et al.*, 2010).

Abnormal miRNA expression has been described in different pathological conditions, such as malignancies, cardiovascular diseases, inflammatory disorders and gynaecological pathologies (Urbich *et al.*, 2008; Sonkoly and Pivarcsi, 2009; Zorio *et al.*, 2009; Qin *et al.*, 2012; Ramón *et al.*, 2012). Some reports have described the miRNA expression profiles in eutopic endometrium and ovarian endometrioma from women with endometriosis (Pan *et al.*, 2007; Pan and Chegini, 2008; Toloubeydokhti *et al.*, 2008; Ohlsson-Teague *et al.*, 2009, 2010; Filigheddu *et al.*, 2010). Nonetheless, these reports studied only a few tissues, and neither angiogenic nor proteolytic factors were detected in different tissues from the same patient.

We previously analysed eight miRNAs related to angiogenesis (angio-miRs) and compared the expression of several angiogenic factors in 41 paired samples of eutopic and ovarian endometrioma tissues to 31 endometrial tissue samples from control women (Ramón *et al.*, 2011). The expression of these selected angio-miRs differed between eutopic endometrium samples and ovarian endometrioma samples. This might influence the expression of angiogenic factors and play a role in the pathogenesis of endometriosis.

The objective of the present study was to analyse the miRNA expression profile in endometriosis and to correlate this profile with several angiogenic and fibrinolytic factors in different endometriotic lesions (ovarian endometrioma, peritoneal lesions and rectovaginal nodules) from the same patient with endometriosis compared with control endometrium. To our knowledge, this is the first study that evaluates the miRNA expression profile in different ectopic lesions and eutopic endometrium from the same patient, and compared these with the miRNA profile of endometrium from control women.

Materials and Methods

Clinical groups

Fifty-one Caucasian women with endometriosis were studied (mean age: 34.0 years, range: 20–45). All women underwent laparoscopic surgical examination of the abdominal cavity and complete excision of endometriotic tissue. The presence of the disease was suspected either by clinically or by ultrasonography and confirmed by surgical findings and post-operative pathological examination. Laparoscopic examination of the abdominal cavity excluded the presence of any other pelvic pathology that could potentially confound the data observed. The main symptom for surgery in this group of patients was abdominal pain (74.5%) and sterility (25.5%).

Thirty-two Caucasian asymptomatic women without endometriosis, who underwent surgery for laparoscopic tubal sterilization, were included in the control group (mean age: 36.4 years, range: 27–45). Absence of the disease was confirmed after surgical examination of the abdominal cavity. Meticulous examination of the peritoneum, ovaries, intestine and diaphragm was performed to detect any typical or atypical endometriotic lesions. Biopsies of suspicious areas for endometriosis were confirmed to be negative in these women.

The menstrual phase was identified according to the day of the reproductive cycle and histological analysis of the endometrium. Whereas 26 (51%) women with endometriosis were in the proliferative phase, 25 (49%) were in the secretory phase of the menstrual cycle. Whereas 15 (47%) controls were in the proliferative phase, 17 (53%) were in the secretory phase of the menstrual cycle. Women in the menstrual phase were excluded from the study.

Patients with irregular menstruation or women who had been pregnant or breastfeeding in the previous 6 months were excluded from the study. None of the women had received hormonal treatment for at least 3 months before the study. Informed consent was obtained from all patients and controls, and the study was approved by the Institutional Review Board.

Tissue extracts

Paired ovarian endometriomas and endometrial biopsies (eutopic endometrium) were obtained from 51 patients with endometriosis. Moreover, peritoneal implants were obtained from 18 of the 51 patients, and rectovaginal nodules were obtained from 20 of the 51 patients with endometriosis. Tissue samples from ovarian endometriomas were macroscopically separated from ovarian tissue and peritoneal implants were excised surgically, avoiding the use of electrocautery in the dissection. Rectovaginal nodules were detected through clinical examination, and infiltration of the rectum was discarded by magnetic resonance imaging or rectal ultrasonography. Specimens were obtained after laparoscopic excision of the rectovaginal nodule, and macroscopic tissue sampling was performed at the core of the lesion. Small lesions (measuring <2 cm) or fragmented lesions were rejected to avoid artefacts caused by manipulation of the tissue during surgery or electrocoagulation. Endometrial biopsies from patients (eutopic endometrium) were performed using an atraumatic endometrial suction cannula. Thirty-two endometrial biopsies from women without endometriosis (control endometrium) were performed using the same procedure.

All samples were rinsed in phosphate-buffered saline, and endometriotic tissues were also evaluated microscopically to confirm the diagnosis. Tissue samples were stored in liquid nitrogen until they were processed.

RNA extraction and quality determination

Total RNA was extracted from endometrial and endometriotic tissues and control endometrial tissues using the mirVana miRNA isolation kit (Ambion, Austin, TX, USA), according to manufacturer's protocol. The RNA concentration and purity were determined using a NanoDrop ND-1000 spectrophotometer (Nanodrop Technologies, Wilmington, DE, USA) in all samples. Following manufacturer's specifications, only samples with A260/A280 ratio of $\sim$2.0 and A260/230 ratio in the range of 1.9–2.2 were considered for inclusion in the study. RNA integrity was analysed using the Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA). We established a RNA integrity number cut-off value of $\geq$7.0 for microarray assays; this was based on the definition used by Ibberson *et al.* (2009) to define good RNA quality.

Analysis of miRNA expression profiles

For each sample, 500 ng of total RNA was labelled with the FlashTag Biotin RNA Labelling Kit (Genisphere, Hatfield, PA, USA) according to manufacturer's recommendations. Briefly, a tailing reaction was carried out at 37°C for 15 min (10 μl of total RNA and spike control oligos mix, 1× reaction buffer, 2.5 mM $MnCl_2$, 1.33 μM ATP and 1 μl Poly A polymerase enzyme), and this was followed by ligation of the biotinylated signal molecule to the target RNA sample at 25°C for 30 min (with the addition of 4 μl of 5× FlashTag Ligation Mix Biotin and 2 μl of T4 DNA Ligase into the 15 μl of reaction mix). Finally, 2.5 μl of stop solution was added to terminate the reaction.

Labelled RNAs were hybridized on Affymetrix GeneChip miRNA 2.0 arrays (Affymetrix, Santa Clara, CA, USA) at 48°C and 60 rpm for 16 h in the presence of total biotin-labelled RNA, hybridization mix, formamide, DMSO, eukaryotic hybridization controls and control oligonucleotide B2. Immediately following hybridization, the arrays were washed and stained with streptavidin–phycoerythrin conjugate in the GeneChip® Fluidics Station 450. Finally, they were scanned using a GeneChip® Scanner 3000 7G.

Microarray data analysis

For data analysis, Affymetrix *.cel data* files were imported into PARTEK Genomic Suite software (PARTEK, St. Louis, MO, USA) and normalized using the Robust Multi-array Analysis (RMA) algorithm. Following the miRNA expression workflow, normalization of data included RMA, background correction, quartile normalization, log2 transformation values and median polish according to the Genisphere indications for the FlashTag Biotin labelling kit. After ANOVA statistical analysis, the miRNA generated lists were used for further analysis including only miRNAs with a *P*-value of <0.05.

Principal component analysis (PCA) was performed in all array data from hybridized samples, so they were reviewed according to their characteristic miRNA expression profiles. Hierarchical clustering representation of differentially expressed miRNA from all studied samples allowed to identify samples with similar of miRNA expression patterns, according to *P*-values and fold-change criteria. Compared with PCA supervised hierarchical clustering represents, only the generated lists of miRNAs that are differentially expressed.

Identification of miRNA target genes by computational tools

The bioinformatic tools mirbase.org, microrna.org, targetscan.org and Diana tools were used to predict the genes targeted by miRNAs expressed differently in different categories: ovarian endometrioma, eutopic endometrium and control endometrium.

Quantification of selected mature miRNAs by quantitative real-time RT–PCR

Differentially expressed miRNAs with targets implicated in angiogenesis, proteolysis or endometriosis selected in microarray studies were tested by real-time quantitative RT-PCR (qRT-PCR) in a larger cohort of samples, including the samples in which microarray experiments were performed (32 control endometrial samples, 51 paired samples of eutopic endometria and ovarian endometrioma, 18 peritoneal implants and 20 rectovaginal nodules of the 51 patients).

The study involved 12 miRNAs (miR-16-5p, -29c-3p, -138-5p, 202-3p, -373-3p, -411-5p, -411-3p, -424-5p, -449b-3p, -556-3p, -636, -935) and the small nucleolar RNA RNU6B, which provided an endogenous control, which was detected stably expressed in quantitative real-time RT–PCR runs.

Quantification was performed by miRCURY LNA™ Universal RT microRNA PCR (Exiqon, USA). This method is based on a universal reverse transcription followed by a real-time PCR amplification with LNA™ enhanced primers. The protocol was performed as outlined in the instruction manual, employing a Light cycler 480 instrument (Roche).

Total protein extraction and quantification

Cytosolic and membrane protein extracts from endometriotic and endometrial tissues were obtained as previously described (Bouchet-Bernet *et al.*, 1996). Total protein concentration for all the tissue extracts was determined using the BCA protein assay (Pierce, Rockford, IL, USA). Fraction-V bovine serum albumin (Sigma-Aldrich, St. Louis, MO, USA) was used for calibration. Samples and standards were analysed in duplicate.

Vascular endothelial growth factor, thrombospondin-1, urokinase plasminogen activator and plasminogen activator inhibitor-1 protein quantification in tissue lysates

Vascular endothelial growth factor (VEGF-A) protein levels were measured using a commercially available ELISA kit (Human VEGF, IBL International, Germany). No cross-reactivity or interference with platelet-derived growth factor was observed. This assay recognizes both human VEGF-A_{165} and VEGF-A_{121} isoforms. The intra-assay and inter-assay variabilities were 4–6 and 7–10%, respectively. The lower detection limit was 15 pg/ml.

Thrombospondin-1 (TSP-1) protein levels were quantified using a commercially available ELISA kit (Human TSP-1, ELISA Development System, DuoSet, RD systems, Minneapolis, MN, USA). No cross-reactivity or interference with TSP-2 or TSP-4 was observed. The intra-assay and inter-assay variabilities were 5–6 and 8–11%, respectively. The lower detection limit was 1.56 ng/ml.

Urokinase plasminogen activator (uPA) protein levels were quantified by a commercially available ELISA (Zymutest uPA, Hyphen Biomed, France), which measures single-chain urokinase (scuPA) and the high-molecular-weight form of uPA (HMW-uPA) with similar efficiency. The intra-assay and inter-assay variation coefficients were 3–5 and 8–11%, respectively. The lower detection limit was 0.12 ng/ml.

Plasminogen activator inhibitor-1 (PAI-1) protein levels were quantified by a commercially available ELISA (Imubind tissue PAI-1, America Diagnostica, USA). The assay detects free and complexed PAI-1 and is insensitive to PAI-2. The intra-assay and inter-assay variation coefficients were 3–4 and 6–8%, respectively. The lower detection limit was ~0.25 ng/ml.

Statistical analysis

All variables were checked for normal distribution by using the Kolmogorov–Smirnov test. Differences in the studied variables between two groups were analysed using the unpaired Student *t*-test or Mann–Whitney U test. Differences in the studied variables among several groups were analysed by a one-way ANOVA test or Kruskal–Wallis test.

miRNA data quantified by real-time qRT-PCR are presented as fold changes relative to the women without endometriosis group (control endometrium = 1). Values are expressed as mean ± SEM.

Levels of significance in correlations between variables were calculated by the bivariate Pearson correlation test. *P*-values of <0.05 (two-tailed) were considered significant. All these tests were performed using the statistical package SPSS Release 20 for Windows (SPSS Inc.).

Results

miRNA expression profiles determined by microarray analysis

The GeneChip miRNA 2.0 Array contains 1105 probes for mature human miRNAs and 1105 probes for pre-miRNAs of these miRNAs (miRBase version 15). Profiling of these non-coding RNAs was completed in seven eutopic endometrial tissues and three ovarian endometrioma tissues from patients, and from endometrial tissues from five healthy controls. Results generated from the three categories using the Affymetrix GeneChip 2.0 were analysed by PCA using Partek Genomic Suite Software. This graphical algorithm showed a relatively close relationship between the expression profiles of control and eutopic endometrium, which were both relatively isolated from ovarian endometrioma (Fig. 1A). When the three sample categories were compared, we found 156 mature miRNAs that were differentially expressed at least 1.3-fold ($P < 0.05$; 79 up-regulated and 77 down-regulated) in ovarian endometrioma or in eutopic endometrium or in both tissues compared with healthy tissue (Supplementary data, Table SI). Supervised hierarchical clustering of differentially expressed miRNAs showed closer expression signatures in control and eutopic endometrium, with ovarian endometrioma clustering separately from control and eutopic endometrium (Fig. 1B).

We next performed an '*in silico*' study of the genes predicted to be targeted by the 156 differentially expressed miRNAs to investigate which of them might regulate the expression of the most important factors involved in angiogenesis and fibrinolysis, or that had been implicated in endometriosis. This analysis led us to select 12 miRNAs for qRT-PCR validation that employed miRCURY LNA Universal RT microRNA PCR to

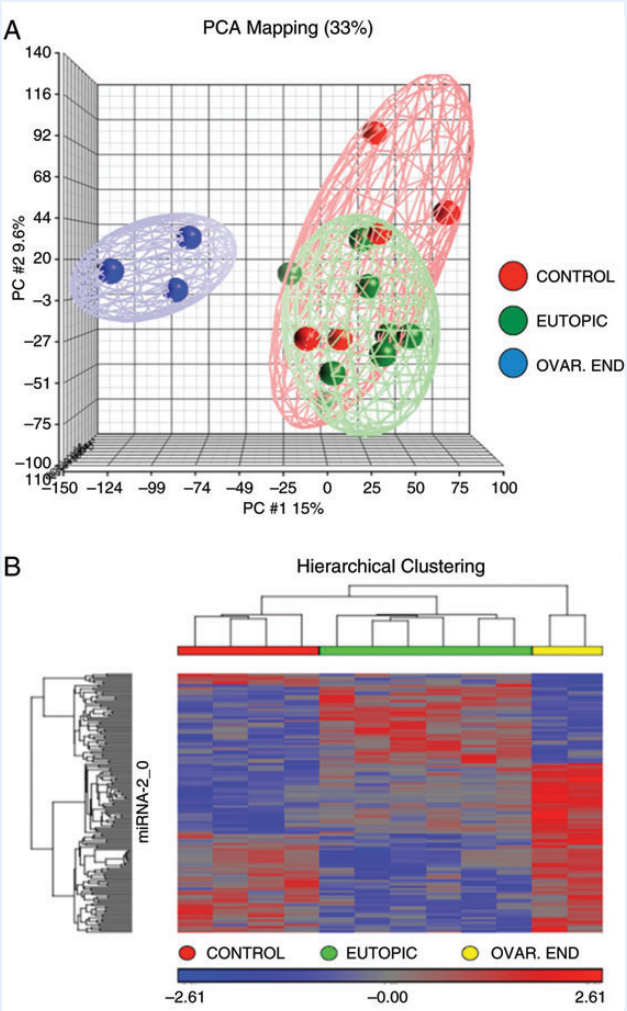

Figure 1 Graphic algorithms showing miRNA expression profiles from endometrial tissues and ovarian endometrioma from patients and control endometrium. (**A**) Principal component analysis applied to the expression of all probes on the Affymetrix GeneChip miRNA 2.0 array. (**B**) Hierarchical cluster analysis of differentially expressed miRNAs from eutopic endometrium, ovarian endometrioma and control endometrial samples.

Table I miRNA microarray expression and targets of miRNA selected for the PCR experiments.

miRNA (v. 15)[a]	miRNA (v. 20)[b]	miRNA sequence 5′–3′	Endometrioma versus control		Eutopic versus control		Target
			Fold-change	*P*-value	Fold-change	*P*-value	
miR-16	miR-16-5p	UAGCAGCACGUAAAUAUUGGCG	−1.06806	0.00073	−1.00307	0.77231	VEGFA, EGFR2, BCL2, FGFR1, COX2
miR-29c	miR-29c-3p	UAGCACCAUUUGAAAUCGGUUA	1.52029	0.00588	1.19676	0.09986	VEGFA, PDGFB-C, THSD4 (TSP-1D4), SERBP1(PAI-1 mRNA binding protein), ADAMTS2, 5–7, 9, 17–19
miR-138	miR-138-5p	AGCUGGUGUUGUGAAUCAGGCCG	1.14975	0.00012	1.49604	0.02856	ADAMTS5, BCL2, TNFF4
miR-202	miR-202-3p	AGAGGUAUAGGGCAUGGGAA	6.16021	0.00339	1.11787	0.49639	THBS1 (TSP-1), GLI1[c], IL6R, MMP1, FGF5, FGF11, IL6, IL10
miR-373*	miR-373-3p	GAAGUGCUUCGAUUUUGGGGUGU	−1.59872	0.01366	−1.5995	0.01087	VEGFA, IL8, ADAMST18, MMP24, TIMP3,ESR1
miR-411	miR-411-5p	UAGUAGACCGUAUAGCGUACG	1.30273	0.01779	1.0831	0.20828	CDH2, ADAMST19
miR-411*	miR-411-3p	UAUGUAACACGGUCCACUAACC	2.41509	0.01738	1.73618	0.01988	ADAMTS1, HIF1α, CDH2
miR-424	miR-424-5p	CAGCAGCAAUUCAUGUUUUGAA	1.13317	0.01904	1.22434	0.17864	VEGFA, IL1, FGF2
miR-449b*	miR-449b-3p	CAGCCACAACUACCCUGCCACU	−2.10914	0.02846	−1.09801	0.40826	MMP-16, IL6R, PDGFRA, PDGFRB
miR-556-3p	miR-556-3p	AUAUUACCAUUAGCUCAUCUUU	−1.37746	0.06692	−1.42387	0.00112	VEGFA, ADAMST1, SERBP1(PAI-1 mRNA binding protein), CDN7
miR-636	miR-636	UGUGCUUGCUCGUCCCGCCCGCA	−1.76706	0.12815	−1.52172	0.04888	ADAMTS14, SERBP1, PDGFRA, FGF12
miR-935	miR-935	CCAGUUACCGCUUCCGCUACCGC	−1.68699	0.84800	−1.4834	0.01543	SERBP1, FGF1

[a]Referred to miRBase database release (version 15).
[b]Referred to miRBase database release (version 20). miRNAs are named in microarray according to miRBase version 16. However, the current classification is referred to miRBase 20 release.
[c]Zhao *et al.* (2013).

examine a larger cohort of samples than used in our initial analysis (Table I). Among the selected miRNAs, six miRNAs (miR-29c-3p, -138, -202-3p, -411-5p, -411-3p, -424-5p) were significantly up-regulated in ovarian endometrioma or in eutopic endometrium compared with the control endometrium, and six miRNAs (miR-16, -373-3p, -449b-3p, -556-3p, -636, -935) were significantly down-regulated in both ovarian endometrioma or eutopic endometrium when compared with their levels in control endometrium.

Quantification of selected miRNAs by real-time qRT-PCR in endometriotic lesions and eutopic endometrial tissues compared with control endometrium

The expression of 12 selected miRNAs was quantified by qRT-PCR in 32 control endometrial samples and 51 paired samples of eutopic endometria and ovarian endometrioma from the same patient. We found that eutopic endometrium expressed significantly less miR-202-3p, miR-424-5p, miR-449b-3p and miR-556-3p than control endometrium (Table II, Fig. 2A–C and E). However, ovarian endometrioma expressed significantly higher levels of miR-29c, -138, -202-3p, -373-3p and -411-5p in comparison with control endometrium (Table II, Fig. 2Dand F–H), whereas miR-449b-3p was significantly less abundant in samples of ovarian endometrioma compared with control endometrium (Table II, Fig. 2B). In the case of miR-202-3p, the up-regulation was near to a 100-fold increase in ovarian endometrioma compared with control endometrium and a 200-fold changes increase compared with with eutopic endometrium (Fig. 2D).

Moreover, these selected miRNAs were quantified in 18 peritoneal and 20 rectovaginal endometriotic lesions from the 51 patients to assess whether or not these miRNAs were dysregulated in these ectopic lesions. Peritoneal implants and rectovaginal lesions showed significantly higher expression of miR-16-5p, -29c-3p, -138-5p, -202-3p, -411-5p, -424-5p and -935 than in control and eutopic endometrium (Table II, Fig. 2A, D and F–H). In contrast, in ovarian endometrioma and rectovaginal lesions, levels of miR-449b-3p were lower than those in control endometrium (Table II, Fig. 2B). Moreover, peritoneal implants as well as eutopic endometrium expressed significantly less miR-556-3p than control endometrium.

miRNAs in endometriotic lesions in comparison to eutopic endometrium

The levels of miR-29c-3p, -138, -411-5p and 424-5p were higher in ovarian endometrioma samples, peritoneal implants and rectovaginal lesions than in eutopic endometrium from patients ($P < 0.001$) (Table II, Fig. 2A, D and F–H). Moreover, levels of miR-202-3p were significantly higher ($P < 0.001$) in ovarian endometrioma, peritoneal implants and rectovaginal lesions than in samples of eutopic endometrium (Table II, Fig. 2D).

Angiogenic and fibrinolytic components in endometriotic lesions and eutopic endometrial tissues compared with control endometrium

Whereas levels of VEGF-A and uPA were significantly higher in eutopic endometrium than in control endometrium (Table III, Fig. 3A and C), no significant differences were observed in TSP-1 levels between eutopic endometrium and control endometrium (Table III, Fig. 3B).

Levels of TSP-1 and PAI-1 were significantly higher ($P < 0.001$) in ovarian endometrioma than in control endometrial tissues (Table III, Fig. 3B and D). Levels of VEGF-A and uPA were not significantly different between ovarian endometrioma and control endometrium (Table III, Fig. 3A and C).

Table II miRNAs levels in tissue extracts from endometrium and endometriotic lesions from women with endometriosis relative to those in endometrium from women without endometriosis (control endometrium).

	Control endometrium, $n = 32$	Eutopic endometrium, $n = 51$	Ovarian endometrioma, $n = 51$	Peritoneal endometriosis, $n = 18$	Rectovaginal endometriosis, $n = 20$
miR-16-5p	1.00 ± 0.08	1.69 ± 0.18	1.72 ± 0.27	6.12 ± 0.70$^{***, ###, \&\&\&}$	10.70 ± 2.50$^{***, ###, \&\&\&}$
miR-29c-3p	1.00 ± 0.15	0.96 ± 0.07	7.67 ± 0.79$^{***, ###}$	10.35 ± 1.43$^{***, ###}$	17.11 ± 3.16$^{***, ###, \&\&\&}$
miR-138-5p	1.00 ± 0.19	2.15 ± 0.54*	4.47 ± 0.71$^{***, ###}$	12.89 ± 2.39$^{***, ###}$	16.03 ± 2.84$^{***, ###, \&\&\&}$
miR-202-3p	1.00 ± 0.16	0.56 ± 0.07**	113.6 ± 14.30 $^{***, ###}$	11.38 ± 5.03$^{*, ###}$	15.92 ± 6.15$^{*, ###, \&\&\&}$
miR-373-3p	1.00 ± 0.11	0.89 ± 0.08	1.58 ± 0.16*	2.18 ± 0.42$^{*, ##}$	1.59 ± 0.50
miR-411-5p	1.00 ± 0.14	0.90 ± 0.08	3.73 ± 0.38$^{***, ###}$	4.28 ± 0.67$^{***, ###, \&\&\&}$	7.53 ± 0.97$^{***, ###, \&\&, \phi}$
miR-411-3p	1.00 ± 0.08	0.90 ± 0.06	1.23 ± 0.13	1.32 ± 0.29*	2.39 ± 0.43$^{**, ###, \&\&, \phi}$
miR-424-5p	1.00 ± 0.14	0.71 ± 0.07*	1.07 ± 0.15$^{\#}$	1.76 ± 0.46$^{*, ##}$	1.89 ± 0.35$^{*, ###, \&}$
miR-449b-3p	1.00 ± 0.15	0.71 ± 0.10*	0.28 ± 0.05$^{***, ###}$	1.07 ± 0.52	0.47 ± 0.10$^{**, \#}$
miR-556-3p	1.00 ± 0.21	0.59 ± 0.09*	0.99 ± 0.17	0.32 ± 0.12$^{**, \&\&}$	1.35 ± 0.53
miR-636	1.00 ± 0.09	1.11 ± 0.11	0.95 ± 0.11	1.76 ± 0.36	1.78 ± 0.59
miR-935	1.00 ± 0.13	0.86 ± 0.008	1.35 ± 0.23	4.95 ± 1.68$^{*, ##, \&}$	3.15 ± 0.61$^{***, ###, \&\&}$

Data are expressed as mean ± SEM. miRNA expression is presented as fold-change relative to women without endometriosis (control endometrium = 1).
Any group versus control endometrium: $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$. Any group versus eutopic endometrium: $^{\#}P < 0.05$; $^{\#\#}P < 0.01$; $^{\#\#\#}P < 0.001$.
Any group versus ovarian endometrioma: $^{\&}P < 0.05$; $^{\&\&}P < 0.01$; $^{\&\&\&}P < 0.001$. Rectovaginal versus peritoneal endometriosis: $^{\phi}P < 0.05$.

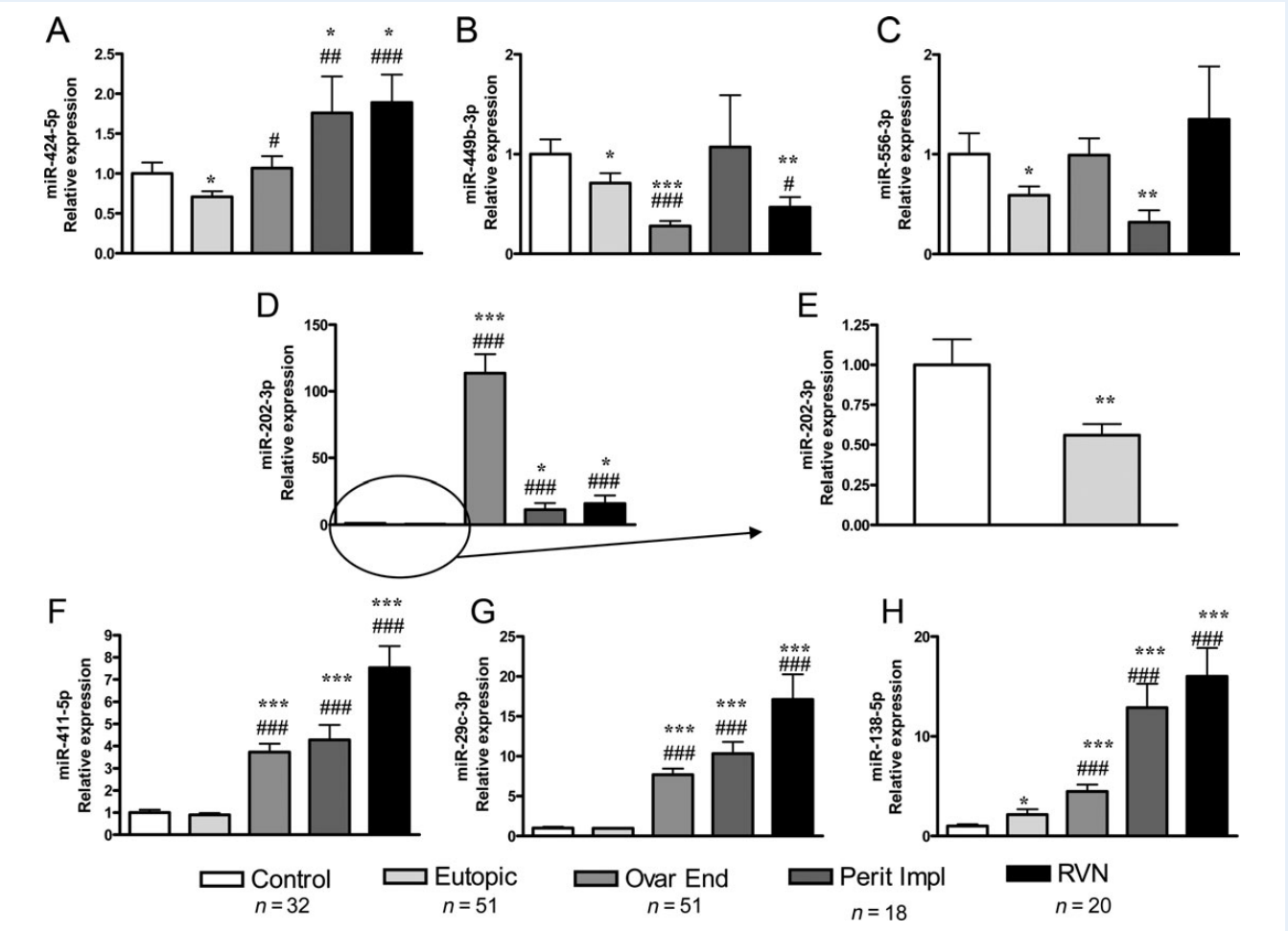

Figure 2 Results from validation by qRT-PCR of miR-424-5p, -449b-3p, -556-3p, -202-3p (down-regulated in eutopic endometrium compared with control endometrium), and miR-202-3p, -411-5p, -29c-3p and -138-5p (up-regulated in endometriotic lesions). (**A**) miR-424-5p. (**B**) miR-449b-3p. (**C**) miR-556-3p. (**D** and **E**) miR-202-3p. (**F**) miR-411-5p. (**G**) miR-29c-3p. (**H**) miR-138-5p. miRNA expression is presented as fold-change relative to control (control endometrium = 1). Data are expressed as mean + SEM. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ versus control endometrium. $^{\#}P < 0.05$; $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$ versus eutopic endometrium. Control: control endometrium ($n = 32$); Eutopic: eutopic endometrium ($n = 51$); Ovar. End: ovarian endometrioma ($n = 51$); Perit. Impl: peritoneal implant ($n = 18$); RVN: rectovaginal nodule ($n = 20$).

Levels of VEGF-A were significantly higher in peritoneal implants than in control endometrium (Table III, Fig. 3A and C). In contrast, rectovaginal lesions showed an increase in TSP-1 ($P < 0.05$) levels and PAI-1 levels ($P < 0.01$) compared with control endometrium (Table III, Fig. 3B and D).

Comparison of miRNAs and protein levels in endometriotic lesions

Peritoneal lesions showed a significant increase in VEGF-A levels and a significant decrease in miR-556-3p compared with the values found in ovarian endometrioma (Tables II and III). Moreover, peritoneal lesions showed a significant decrease in TSP-1 levels in comparison to ovarian endometrioma and rectovaginal lesions (Table III).

In rectovaginal nodules, a significant decrease in TSP-1 was observed in comparison to ovarian endometrioma (Table III). Moreover, rectovaginal lesions showed a significant increase in miR-411-5p and miR-411-3p compared with ovarian endometrioma and peritoneal lesions (Table II).

Peritoneal and rectovaginal lesions also showed a significant increase in miR-138-5p and miR-935 in comparison to ovarian endometrioma (Table II).

Correlation between differentially expressed miRNAs and angiogenic and fibrinolytic components in tissue extracts

Given that miRNAs often inhibit the translation of their target mRNA, we studied the correlation between miRNA and protein levels. We found a significant inverse correlation between miR-424-5p expression and VEGF-A protein levels ($r = -0.380$, $P = 0.006$) in eutopic endometrium from women with endometriosis and a significant inverse correlation between miR-449b-3p and TSP-1 protein levels ($r = -0.314$, $P = 0.02$) in ovarian endometrioma.

Moreover, there was a significant inverse correlation between miR-424-5p and VEGF-A protein levels in control endometrium ($r = -0.352$, $P < 0.05$).

Table III Vascular endothelial growth factor-A , thrombospondin-1 , urokinase and plasminogen activator inhibitor type 1 protein levels in tissue extracts from endometrium and endometriotic lesions from women with endometriosis and endometrium from women without endometriosis (control endometrium).

	Control endometrium, $n = 32$	Eutopic endometrium, $n = 51$	Ovarian endometrioma, $n = 51$	Peritoneal endometriosis, $n = 18$	Rectovaginal endometriosis, $n = 20$
VEGF-A protein (pg/mg)	94 ± 11	218 ± 34**	118 ± 16$^{\#\#}$	445 ± 60***, $^{\#\#\#, \&\&\&}$	316 ± 50**, $^{\#, \&\&}$
TSP-1 protein (ng/mg)	55 ± 8	102 ± 17	941 ± 102***, $^{\#\#\#}$	131 ± 74$^{\&\&\&}$	329 ± 99***, $^{\#, \&\&, \phi}$
uPA protein (ng/mg)	1.08 ± 0.09	1.71 ± 0.21*	1.63 ± 0.23	0.20 ± 0.05$^{\#\#\#, \&\&\&}$	0.31 ± 0.08$^{\#\#, \&\&}$
PAI-1 protein (ng/mg)	2.17 ± 0.30	4.36 ± 0.73*	18.58 ± 2.95***, $^{\#\#\#}$	3.75 ± 1.66*, $^{\&\&\&}$	5.36 ± 1.17***, $^{\&\&}$

Data are expressed as mean ± SEM.
Any group versus control endometrium: $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$.
Any group versus eutopic endometrium: $^{\#}P < 0.05$; $^{\#\#}P < 0.01$; $^{\#\#\#}P < 0.001$.
Any group versus ovarian endometrioma: $^{\&}P < 0.05$; $^{\&\&}P < 0.01$; $^{\&\&\&}P < 0.001$.
Rectovaginal versus peritoneal endometriosis: $^{\phi}P < 0.05$.

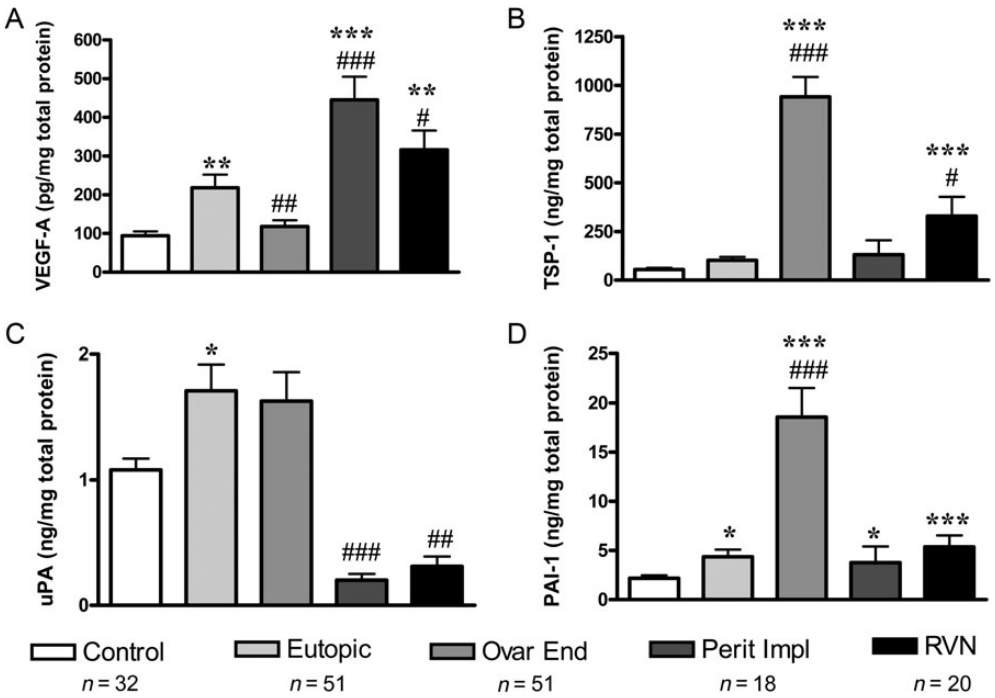

Figure 3 Protein levels of vascular endothelial growth factor-A (VEGF-A), thrombospondin-1 (TSP-1), urokinase plasminogen activator (uPA) and plasminogen activator inhibitor-1 (PAI-1) from different tissues. (**A**) VEGF-A. (**B**) TSP-1. (**C**) uPA. (**D**) PAI-1. Data are expressed as mean + SEM. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ versus control endometrium. $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$ versus eutopic endometrium. Control: control endometrium ($n = 32$); Eutopic: eutopic endometrium ($n = 51$); Ovar. End: ovarian endometrioma ($n = 51$); Perit. Impl: peritoneal implant ($n = 18$); RVN: rectovaginal nodule ($n = 20$).

Influence of the phase of menstrual cycle on miRNA levels

To evaluate the possibility that the phase of the menstrual cycle might affect the expression of miRNAs, we performed the statistical analysis on data collected at different phases of the cycle. No significant differences were observed between the secretory and proliferative phases for the different groups for any of the miRNAs studied.

Discussion

In the present study, we identified 156 mature miRNAs (79 up-regulated and 77 down-regulated) that were differentially expressed at least 1.3-fold in ovarian endometrioma and/or eutopic endometrium compared with control endometrial tissues applying microarray technology to obtain miRNA expression profiles. Supervised hierarchical clustering and PCA showed that whereas control and eutopic endometrium had

similar miRNA profiles, ovarian endometrioma showed different expression patterns. The '*in silico*' study of the target genes for those differentially expressed miRNAs enabled us to select 12 miRNAs to validate by qRT-PCR in all the samples. Patient endometrium showed higher VEGF-A levels and lower expression of miR-202-3p and miR-449b-3p compared with control endometrium. In addition, ovarian endometrioma showed significantly higher expression of the angiogenic inhibitor TSP-1 and lower expression of miR-449b-3p than control endometrium. Moreover, miR-29c-3p and miR-202-3p were more abundant in endometriotic tissues than control and patient endometrium.

To our knowledge, this is the first study to evaluate the expression profiles of miRNAs in different ectopic lesions and eutopic endometrium from the same patient, and to compare this with endometrium from control women.

miRNA expression levels of eutopic and ectopic lesions from women with endometriosis have been described recently (Pan *et al.*, 2007; Toloubeydokhti *et al.*, 2008; Burney *et al.*, 2009; Ohlsson-Teague *et al.*, 2009, 2010; Filigheddu *et al.*, 2010; Ramón *et al.*, 2011; Laudanski *et al.*, 2013), but only a few studies have reported differences in miRNA expression in the eutopic endometrium from women with and without endometriosis (Pan *et al.*, 2007; Toloubeydokhti *et al.*, 2008; Burney *et al.*, 2009; Laudanski *et al.*, 2013).

The present study revealed that miR-202-3p expression was significantly lower in eutopic endometrium samples and significantly higher in ovarian endometrioma samples when compared with control endometrium. Recently, it has been reported (Zhao *et al.*, 2013) that miR-202-3p down-regulates the expression of the glioma-associated oncogene homolog 1 (GLI1) transcription factor. This protein regulates the expression of genes involved in proliferation, migration, invasion and angiogenesis through its effects on regulators, such as BCL-2, CD24, metalloproteinase-2 (MMP-2) and MMP-9 (Carpenter and Lo, 2012). GLI1 also regulates the transcription of VEGF-A (Cao *et al.*, 2012; Carpenter and Lo, 2012; Santoni *et al.*, 2013; Zhao *et al.*, 2013). In addition, the anti-apoptotic protein BCL-2 is overexpressed in the eutopic endometrium of women with endometriosis (Burney *et al.*, 2009; Burney and Giudice, 2012). This BCL-2 overexpression could be modulated by miR-202-3p by regulating the expression of the GLI1 transcription factor. Therefore, the significantly reduced levels of miR-202-3p observed in eutopic endometrium compared with control endometrium could contribute to the increased levels of VEGF-A observed in this tissue. In contrast, the increase of miR-202-3p observed in ovarian endometrioma might account, at least in part, for the low angiogenic activity and low-invasive capacity of this tissue. These findings suggest that dysregulation of miRNA during endometriosis might play a pivotal role establishing endometriotic lesions by affecting different physiological processes.

miR-424-5p targets VEGF-A and plays an important role in down-regulating the angiogenic activity of this protein (Wang and Olson, 2009; Chamorro-Jorganes *et al.*, 2011). The lower levels of miR-424-5p and higher levels of VEGF-A protein in eutopic endometrium samples from patients than samples from controls, as well as the inverse correlation between miR-424-5p and VEGF-A protein levels observed in the present study, suggest that this miRNA might account, at least in part, for the higher VEGF-A levels observed in eutopic endometrium from patients compared with controls. In the present report, lower levels of miR-556-3p and higher levels of VEGF-A have been found in eutopic endometrium and peritoneal implant samples from patients when compared with endometrium from controls. Because one of the targets of miR-556-3p is VEGF-A, this miRNA also might contribute for the increase in VEGF-A levels in eutopic endometrium and peritoneal lesions. These increased angiogenic properties of endometrium might be essential to the initial formation of active peritoneal implants and the development of the vascular network that facilitates the growth and invasion of the ectopic tissue.

It has been reported that miR-449b-3p is down-regulated in ectopic endometrium compared with control endometrium (Hawkins *et al.*, 2011). Our results confirm these findings in a larger number of samples from women with and without endometriosis. Others also reported decreased function of several miRNAs, including miR-449b-3p, and overexpression of miR-204 in endometrial cancer cells compared with healthy endometrial cells; these changes repress migration, invasion and extracellular matrix adhesion in endometrial cancer cells (Chung *et al.*, 2012). The reduced level of miR-449b-3p in ovarian endometrioma relative to healthy tissue might account for the low-invasive capability of these ectopic tissues and the frequent clinical finding that preserved normal tissue is located in the vicinity of the endometriotic lesion.

Remodelling of extracellular matrix protein plays a critically important role in the establishment of endometriotic lesions. Abnormal expression of components of metalloproteinase systems at the mRNA level has been reported in both the endometrium and endometriotic tissue of women affected by endometriosis (Ramón *et al.*, 2005; Klemmt *et al.*, 2007). The present study indicated that miR-29c-3p is more abundant in endometriotic tissues (ovarian endometrioma, peritoneal lesions and rectovaginal nodule) than in healthy endometrial tissue. Given that miR-29c-3p targets different extracellular matrix genes, our results support previous studies that suggest a potential role for a network of miRNAs in the remodelling process that leads to implantation of endometrial tissue outside the uterus and to the formation of endometriotic lesions (Ohlsson-Teague *et al.*, 2009, Filigheddu *et al.*, 2010; Hawkins *et al.*, 2011).

miR-138-5p has been related to several biological processes, including proliferation, migration, invasion and the epithelial to mesenchymal transition in various cancers (Liu *et al.*, 2011; Chakrabarti *et al.*, 2013; Qiu *et al.*, 2013; Wang *et al.*, 2013). It has been postulated that, similar to tumoural metastasis, the epithelial to mesenchymal transition may be a key mechanism that underlies the induction of peritoneal invasion in endometriosis (Matsuzaki and Darcha, 2012; Young *et al.*, 2013). In the present report, we found that miR-138-5p is up-regulated in all studied ectopic lesions. Hence, we hypothesized that miR-138-5p regulates a transition in the cellular morphology to a mesenchymal phenotype.

Endometriosis seems to be a progressive disease and a reduction in the angiogenic activity of endometriotic lesions has been observed in advanced stages (Nisolle *et al.*, 1993). Peritoneal lesions showed higher VEGF-A levels and lower miR-556-3p levels than ovarian endometrioma. On the other hand, ovarian endometrioma showed higher TSP-1 levels and lower VEGF-A levels than peritoneal implants. These results suggest that peritoneal endometriotic implants are lesions with a high potential of angiogenesis and invasion of extracellular matrix, while ovarian endometriomas are lesions with low capability of remodelling the surrounding tissue.

There are defined clinical differences between different types of lesions in endometriosis. While rectovaginal nodules are formed of fibrotic tissue that infiltrate deeply in the retroperitoneum or the pelvic viscera, ovarian endometriomas are cystic structures that grow inside the ovary

respecting surrounding ovarian parenchyma. Peritoneal implants are a wide variety of lesions with typical or atypical appearance. Red active peritoneal implants are characterized by neovascularization an adhesion formation. These biological differences might explain a different expression in VEGF-A, TSP-1 and several studied miRNAs depending on the site of endometriosis.

Further functional studies are necessary to confirm the specific targets of the differently expressed miRNAs in endometrial and endometriotic tissues. Nonetheless, our findings point out that these molecules are attractive candidates in the search for novel diagnostic biomarkers to guide therapeutic interventions to treat endometriosis.

Supplementary data

Supplementary data are available at http://humrep.oxfordjournals.org/.

Authors' roles

A.B.-B. developed the idea for the paper, participated in study design, performed analyses and interpretation of data and wrote the manuscript. J M.-A performed analyses and interpretation of data. J.G, participated in study design, has provided patients of the study and have contributed analysis and interpretation of data and critical revision. D.S.-I. performed array analysis and interpretation of data. F.E. contributed to study design, interpretation of data and critical revision. A.E. developed the idea for the paper, formulated the study design, participated in analysis and interpretation of data and wrote the manuscript. J. G.-E. developed the idea for the paper, formulated the study design, have provided patients of the study and have contributed analysis and interpretation of data and critical revision, and wrote the manuscript. All authors have approved the final version of the manuscript.

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Conflict of interest

None declared.

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Peritoneal Fluid Reduces Angiogenesis-Related MicroRNA Expression in Cell Cultures of Endometrial and Endometriotic Tissues from Women with Endometriosis

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Abstract

Endometriosis, defined as the presence of endometrium outside the uterus, is one of the most frequent gynecological diseases. It has been suggested that modifications of both endometrial and peritoneal factors could be implicated in this disease. Endometriosis is a multifactorial disease in which angiogenesis and proteolysis are dysregulated. MicroRNAs (miRNAs) are small non-coding RNAs that regulate the protein expression and may be the main regulators of angiogenesis. Our ***hypothesis*** is that peritoneal fluid from women with endometriosis could modify the expression of several miRNAs that regulate angiogenesis and proteolysis in the endometriosis development. The ***objective*** of this study has been to evaluate the influence of endometriotic peritoneal fluid on the expression of six miRNAs related to angiogenesis, as well as several angiogenic and proteolytic factors in endometrial and endometriotic cell cultures from women with endometriosis compared with women without endometriosis.

Methods: Endometrial and endometriotic cells were cultured and treated with endometriotic and control peritoneal fluid pools. We have studied the expression of six miRNAs (miR-16, -17-5p, -20a, -125a, -221, and -222) by RT-PCR and protein and mRNA levels of vascular endothelial growth factor-A, thrombospondin-1, urokinase plasminogen activator and plasminogen activator inhibitor-1 by ELISA and qRT-PCR respectively.

Results: Control and endometriotic peritoneal fluid pools induced a significant reduction of all miRNAs levels in endometrial and endometriotic cell cultures. Moreover, both peritoneal fluids induced a significant increase in VEGF-A, uPA and PAI-1 protein levels in all cell cultures without significant increase in mRNA levels. Endometrial cell cultures from patients treated with endometriotic peritoneal fluid showed lower expression of miRNAs and higher expression of VEGF-A protein levels than cultures from controls. In ***conclusion***, this ''in vitro'' study indicates that peritoneal fluid from women with endometriosis modulates the expression of miRNAs that could contribute to the angiogenic and proteolytic disequilibrium observed in this disease.

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Introduction

Endometriosis, which is defined by the presence of endometrial tissue outside of the uterine cavity, is one of the most common gynaecologic disorders causing pelvic pain and infertility [1–3]. Despite the high prevalence and incapacitating symptoms of endometriosis, the precise pathogenic mechanisms of this condition remain unsolved. Sampson's theory of retrograde menstruation is, by far, the most widely accepted [4]. However, although this theory explains the migration of endometrial fragments to ectopic locations, additional steps are required for the development of endometriotic implants [5]. The establishment of these lesions is accompanied by inflammation, neoangiogenesis and subsequent fibrosis, accounting for the symptoms described [6].

Endometriosis is a multifactorial disease in which endometrial and peritoneal factors such as those related to angiogenesis and proteolysis may be involved [7–9]. According to that, components of peritoneal fluid have arisen as an important field of study, provided that ectopic lesions located in the pelvic peritoneum are completely submerged in this fluid [10–13]. Moreover, previous studies have reported that the peritoneal fluid from women with endometriosis induces cell proliferation *in vitro*, although the

mechanism underlying this effect has not further been investigated and remains unknown [13–14].

Angiogenesis may play an important role in the pathogenesis of endometriosis. Endometriotic implants require neovascularization to proliferate, invade the extracellular matrix, and establish an endometriotic lesion, similar to tumour metastases [6–7,15–17]. Several studies, including ours, have reported an increase in vascular endothelial growth factor (VEGF-A) levels in endometriosis, which has been suggested to be an important angiogenic factor playing a major role in the progression of the disease [18–23]. Thrombospondin-1 (TSP-1), an inhibitor of angiogenesis, may also be involved in pathologies of the female reproductive tract such as endometriosis, in which vessel formation occurs [23–25].

Moreover, we have observed an increase in VEGF-A levels, and proteolytic factors, like urokinase plasminogen activator (uPA) and metalloproteinase-3 (MMP-3), in peritoneal fluid from patients with endometriosis in comparison with women without the disease [9,23]. These results suggest that both tissue factors of the endometrium from women with endometriosis as well as peritoneal factors enhance the angiogenic and proteolytic capability of ectopic tissue to facilitate the implantation process.

According to previous reports, microRNAs (miRNAs) may be the main regulators of angiogenesis [26–30]. miRNAs are 21–22 nucleotide non-coding RNAs that regulate gene expression and play fundamental roles in biological processes. These small molecules bind to target mRNAs and mediate translational repression and/or mRNA degradation [31]. Functional analysis of miRNAs has revealed their significant regulatory influence on the expression of target genes involved in both physiological and pathological conditions [32–36]. Aberrant miRNA expression is associated with human diseases such as cancer, cardiovascular disorders, inflammatory diseases, and gynaecological diseases [37–39]. Emerging data have described a different molecular environment [40–41] and an altered miRNA expression in pathologic endometrium in comparison with normal endometrium [32,42–45].

As we have previously described [46], VEGF-A protein levels were significantly higher in eutopic endometrium from patients than in endometrium from control women. In addition, when different endometriotic lesions were compared, VEGF-A protein levels were significantly upregulated in peritoneal lesions in comparison with ovarian or rectovaginal endometriotic lesions. In this same study miRs -16, -17-5p, -20a, -125a, -221 and -222 were quantified in different endometriotic lesions and it was demonstrated that different lesions expressed different levels of these miRNAs. Therefore, we selected these six miRNAs due to their distinct expression in different endometriotic lesions and due to "in silico" studies suggesting that these miRNAs regulate not only VEGF-A expression but other angiogenic factors [32,44,47–50].

Primary cell cultures are a useful tool for studying the response of stromal cells to peritoneal fluid components under controlled conditions. We previously studied the influence of control and endometriotic peritoneal fluid on angiogenic and proteolytic systems in endometrial cell cultures from women with and without the disease [9]. However, the effect of peritoneal fluid on angiogenesis-related miRNA expression and its correlation with angiogenesis and proteolysis in endometrial and endometriotic cell cultures have not been previously studied.

Our hypothesis is that peritoneal fluid components from women with endometriosis may alter miRNA expression in stromal cells from endometrial fragments migrated to peritoneum. Moreover, this altered miRNA expression could contribute to changes of angiogenic and proteolytic components in endometrial tissue and play a role in the establishment of the endometriotic implants.

Therefore, the aim of the present study was to investigate the effect of endometriotic peritoneal fluid on angiogenesis-related miRNA expression in endometrial cell cultures from women with endometriosis compared to control endometrial and endometriotic cell cultures and to assess whether peritoneal fluid modifies the expression of angiogenic and proteolytic factors by miRNA action.

Materials and Methods

Ethics Statement

Written informed consent was obtained from all patients and controls, and the study was approved by the Ethical Committee from Hospital Universitario y Politécnico La Fe, Valencia, Spain (#2008/0111).

Clinical Groups

Patients. Caucasian women with moderate or severe endometriosis (stages III-IV, revised ASRM classification system, 1997) [51] were studied. Complete excision of the ovarian endometrioma (endometriotic tissue) was performed. The diagnosis of endometriosis was confirmed by anatomopathological examination of all specimens obtained. Endometrial biopsies (patient endometrial tissue) from women with moderate or severe endometriosis were performed using a cornier device without curettage, which takes the functional layer.

Controls. Normal endometrial tissues were obtained from fertile women without endometriosis who underwent surgery for tubal sterilization (75%) and pelvic pain (25%). The absence of endometriosis was confirmed by meticulous examination of the pelvic and extrapelvic peritoneum, ovaries, intestine, and diaphragm to detect typical or atypical endometriotic lesions. Biopsies of potential areas of endometriosis were confirmed to be negative in these women. Other gynaecologic pathologies such as adhesions or ovarian or uterine masses were also confirmed to be negative in this control group by preoperative gynaecologic ultrasound and systematic laparoscopic examination of the abdominal cavity during the intervention.

Both peritoneal fluids, from control and patients, were collected immediately after the establishment of the pneumoperitoneum and before laparoscopic manipulation. The peritoneal fluids were centrifuged at $1,500 \times g$ for 30 min at 4°C, filtered through a 0.2-μm pore size membrane, and stored at −80°C.

Women affected by menorrhagia or hypermenorrhea or women who had been pregnant or breast feeding during the previous 6 months were excluded from the study. None of the women had received any form of hormone therapy for at least 3 months before the study.

Tissue Samples

Endometrial tissue (patient endometrial tissue) from 11 women with moderate or severe endometriosis (stages III-IV) (mean age 32.4 years; range 19–40), ovarian endometrioma (endometriotic tissue) from 11 women with moderate or severe endometriosis (stages III-IV) (mean age 30.5 years; range 19–42) and control endometrial tissue from 8 women without the disease (mean age 36.1 years; range 24–43) were obtained for stromal cell isolation.

No statistical significant differences in the age of the groups were observed (patient endometrial *vs* control endometrial tissue, $P = 0.312$; endometriotic tissue *vs* control endometrial tissue, $P = 0.116$).

Peritoneal Fluid Pools

10 peritoneal fluids from women with endometriosis (endometriotic peritoneal fluid pool) (mean age 33.1 years, range 27–39) and 10 peritoneal fluids from fertile women without endometriosis (control peritoneal fluid pool) (mean age 37.2, range 21–47) in the proliferative phase of the menstrual cycle were thawed and pooled.

Primary Cell Culture of Stromal Cells from Endometrial and Endometriotic Tissues

Primary cultures of endometrial cells were prepared as previously described [52] with minor modifications [9]. Endometrial biopsies were collected in PBS containing 50 U penicillin/mL and 50 µg streptomycin/mL (Sigma) and rinsed to remove blood cells, stored at 4°C and processed within 2–18 h. No significant correlations ($P>0.05$) between the sample processing times and the studied parameters were observed in the different groups.

Tissues were cut into 1 mm^3 pieces and incubated at 37°C for 60 minutes in the presence of collagenase (2.5 mg/mL, Sigma). Dissociated tissues were filtered through a nylon sieve to remove undigested material. Purity of the endometrial stromal cells was higher than 95%, as evaluated by positive cellular staining for vimentin (stromal and epithelial cells) and negative cellular staining for cytokeratin (epithelial cells) and CD68 (macrophages), as previously described [9,51]. Cell suspension was centrifuged at 550×g for 5 minutes and the pellet resuspended in DMEM-F12 phenol red-free medium containing 10% fetal bovine serum (FBS, Invitrogen), 50 U penicillin/mL and 50 µg streptomycin/mL (Sigma). Cell viability, assessed by trypan blue exclusion test, was higher than 95%. Then, stromal cells were plated and maintained in a humidified atmosphere of 5% CO_2 and 95% air at 37°C. The culture medium was renewed every 2 days until the cell monolayer reached confluence, and then cells were subcultured. Cells were detached using 0.25% trypsin/0.02% EDTA (Gibco BRL, Paisley, UK) at 37°C and seeded at a density of 270000 cells/well in 12-well plates. Confluent cell cultures in passage 2 were used for experimental assays.

Exposure to Peritoneal Fluid Pools

As previously described [9], preconditioning experiments to optimize the concentration of peritoneal fluid to add to endometrial cell cultures were performed. Peritoneal fluid up to 25% final concentration had no effect on cell viability, whereas higher concentrations presented toxicity. When endometrial cells in culture were treated with 0%, 10% and 25% peritoneal fluid in serum-free medium, VEGF and uPA levels augmented with increasing peritoneal fluid concentration [9]. Thereafter, endometrial cells were treated with 25% peritoneal fluid for 4 hours in all subsequent experiments.

Cells were pre-conditioned for 4 hours in FBS-free medium, shifted to FBS-free medium (ØPF) or supplemented with 25% of peritoneal fluid pools from controls (CPF) and patients (EPF), and incubated for 4 additional hours. Subsequently, cell culture supernatants were collected and aliquoted on ice, and stored at −80°C for protein quantification. RNA was obtained from cell culture extracts and stored at −80°C until use. All experiments were performed in triplicate.

To evaluate the influence of peritoneal fluid pool treatments on the expression of all studied parameters, we calculated the difference between the levels obtained with and without peritoneal fluid exposition of cell cultures from the same patient samples. Then, we correlated results from miRNAs levels with angiogenic and proteolytic factors to determine the relationship among these parameters.

RNA Extraction

Total RNA from primary cell cultures were extracted using the mirVana miRNA isolation kit (Ambion, USA), according to the manufacturer's protocol. Yield and purity of RNA were measured using a NanoDrop ND-1000 spectrophotometer (Nanodrop Technologies).

Quantification of mRNAs

One microgram of total RNA was treated with DNase I (Invitrogen, Carlsbad, CA) and reverse transcribed into first-strand cDNA by using Superscript RNase H^- (Invitrogen) with an oligo $(dT)_{15}$ primer (Promega, USA). cDNA was stored at −20°C until subsequent study. Analysis of VEGF-A, TSP-1, uPA, PAI-1 and β-actin mRNA expression was performed in a Light Cycler termocycler, using version 3.5 software (Roche Molecular Biochemicals, Germany). The specific primers used for amplification of VEGF-A, TSP-1 and β-actin, the reaction mixture, and the PCR conditions were performed as previously described [43].

Quantification of Mature miRNAs

For this study we selected a set of six miRNAs involved in angiogenesis (miR-16, -17-5p, -20a, -125a, -221 and -222) and RNU6B (small nuclear RNA) as endogenous control.

Quantification using the TaqMan MicroRNA Assays was done using two-step RT-PCR in total RNA samples. This method quantifies exclusively mature miRNAs but not their precursors. In the reverse transcription, cDNA is reverse transcribed from total RNA samples using specific miRNA primers from the TaqMan MicroRNA Assays and reagents from the TaqMan® MicroRNA Reverse Transcription Kit (Applied Biosystems, Foster City CA). Reactions were performed in an Eppendorf Mastercycler Thermocycler following the manufacture's protocol. Then, PCR products were amplified from cDNA samples using the Taqman MicroRNA Assay and the TaqMan® Universal PCR Master Mix (Applied Biosystems, USA).

Relative quantification of miRNA expression was calculated with the $2^{-\Delta\Delta Ct}$ method (Applied Biosystems user bulletin no. 2), using RNU6B as endogenous control. Data are presented as fold change relative to the mean of control endometrial cell culture without peritoneal fluid exposure (control endometrial culture = 1).

Determination of Angiogenic and Proteolytic Factors

VEGF-A protein level was measured using a commercially available ELISA (Human VEGF, IBL International, Germany). No cross-reactivity or interference with platelet derived growth factor was observed. This assay recognizes human VEGF-A_{165} and VEGF-A_{121} isoforms. The intra-assay and inter-assay variation coefficients were 4–6% and 7–10%, respectively.

TSP-1 protein level was quantified using a commercially available ELISA (Human TSP-1, ELISA Development System, DuoSet, RD systems, USA). No cross-reactivity or interference with TSP-2 or TSP-4 was observed. The intra-assay and inter-assay variation coefficients were 5–6% and 8–11%, respectively.

uPA protein levels were quantified by a commercially available ELISA (Zymutest uPA, Hyphen Biomed, France), which measures single-chain urokinase (scuPA) and the high weight molecular form of uPA (HMW-uPA) with similar efficiency. The intra-assay and inter-assay variation coefficients were 3–5% and 8–11%, respectively.

PAI-1 protein levels were quantified by a commercially available ELISA (Imubind tissue PAI-1, America Diagnostica,

USA). The assay detects free and complexed PAI-1 and is insensitive to PAI-2. The intra-assay and inter-assay variation coefficients were 3–4% and 6–8%, respectively.

VEGF, uPA, TSP1 and PAI-1 protein levels were determined in culture supernatants and in peritoneal fluid pools. The protein amounts released to the culture medium by cells incubated with peritoneal fluid pools were calculated by subtracting VEGF, TSP1, uPA and PAI-1 contents in the peritoneal fluid pool to the total levels obtained in supernatants.

Statistical Analysis

All the studied parameters showed a normal distribution. Values were expressed as mean ± standard error of the mean (SEM). Differences between means were analyzed by one-way ANOVA test. When significant *P* values were observed, post-hoc analyses were performed using Bonferroni test. Differences between studied variables in the endometrial cells from women with endometriosis vs women without endometriosis for the same treatment were analyzed by unpaired Student t-test. Correlations between variables were calculated by the bivariate Pearson correlation test. *P* values <0.05 (two-tailed) were considered significant. Statistical tests were performed using the statistical package SPSS Release 20 for Windows (SPSS Inc.).

Results

Confluent cultures of stromal cells from patient and control endometrial tissues and endometriotic tissues were treated with serum-free medium containing control or endometriotic peritoneal fluid pools (25% final concentration) or without peritoneal fluid pools (0% final concentration) for 4 hours. Figures 1–4 show levels of different parameters measured in all experimental conditions.

Effect of Peritoneal Fluid on VEGF-A and TSP-1 Levels of Endometrial Cell Cultures from Patient and Control Endometrial Tissues and Endometriotic Tissues (Figure 1)

Control and endometriotic peritoneal fluids significantly enhanced VEGF-A protein levels in all tissue cultures when compared with the corresponding cell culture without peritoneal fluid (Fig 1A). However, peritoneal fluid pools did not significantly modify mRNA expression of VEGF-A (Fig. 1B). The highest VEGF-A protein level was observed in endometrial and endometriotic cell cultures from women with endometriosis treated with the endometriotic peritoneal fluid pool (Fig.1A).

In addition to VEGF-A, which is the most important inductor of angiogenesis, we also studied the main inhibitor of angiogenesis, TSP-1. Treatment of endometrial and endometriotic cell cultures with peritoneal fluid did not significantly modify TSP-1 expression (Fig 1C and 1D).

In the absence of peritoneal fluid exposure, a significant increase ($P<0.05$) in VEGF-A protein levels was observed in endometriotic cell cultures (52.90 ± 16.45 pg/ml) and patient endometrial cell cultures (37.14 ± 11.22 pg/ml) in comparison with control endometrial cell cultures (1.23 ± 0.07 pg/ml) (Fig 1 A).

Effect of Peritoneal Fluid on uPA and PAI-1 Levels of Endometrial Cell Cultures from Patient and Control Endometrial Tissues and Endometriotic Tissues (Figure 2)

Control and endometriotic peritoneal fluid pools induced a similar significant increase in uPA and PAI-1 protein expression (Fig 2A, Fig 2C) without significantly modifying mRNA levels in primary cell cultures of endometrial and endometriotic stromal cells (Fig 2B, Fig 2D).

Effect of Peritoneal Fluid on the Level of miRNAs Related to Angiogenesis (Figure 3)

We selected 6 miRNAs that could be involved in the regulation of angiogenic factors (miR-16, -17-5p, -20a, -125a, -221 and –222).

Exposure of control endometrial cells to peritoneal fluid (Figure 3, white bars), reduced the level of the six miRNAs studied. Exposure of patient endometrial tissue (grey bars) and endometriotic tissue (black bars) cells to peritoneal fluid also reduced miRNA levels, but this reduction was not always statistically significant. The highest miRNA decrease was observed after exposure to endometriotic peritoneal fluid in all cases. Results are expressed as fold change relative to the mean of cell cultures from control endometrium without peritoneal fluid treatments.

Correlation between Changes in miRNA Expression and Changes in Angiogenic and Fibrinolytic Parameters after Treatment with Peritoneal Fluid Pools

To evaluate the influence of peritoneal fluid on the response of the angiogenic system we calculated the difference between each treatment and the basal expression of each studied parameter. We correlated the difference observed in angiogenic or proteolytic factors with the difference observed in miRNA expression after peritoneal fluid pool treatments.

A significant inverse correlation was observed between changes in VEGF-A protein and miR-16 levels in eutopic endometrial and endometriotic cell cultures from women with endometriosis after treatment with peritoneal fluid ($r=-0.525$, $P=0.018$ and $r=-0.733$, $P=0.001$, respectively) (Figure 4). Furthermore, a significant inverse correlation was observed between changes in VEGF-A protein and miR-17-5p ($r=-0.739$, $P=0.001$), miR -20a ($r=-0.676$, $P=0.001$), miR-125a ($r=-0.567$, $P=0.01$) and miR-222 ($r=-0.494$, $P=0.037$) levels in endometriotic cell cultures from women with endometriosis after treatment with peritoneal fluid.

Finally, assessment of the changes in expression of angiogenic and proteolytic factors in response to peritoneal fluid revealed a significant positive correlation between VEGF-A and uPA levels in control endometrial cultures with and without peritoneal fluid exposure ($r=0.719$, $P=0.001$).

Discussion

This study evaluates the influence of peritoneal fluid from women with or without endometriosis on the expression of six miRNAs that modulate angiogenesis, as well as several angiogenic and proteolytic factors, in endometriotic and endometrial cell cultures from women with and without endometriosis.

Several studies have indicated that endometrium and peritoneal fluid from women with endometriosis have different expression patterns of several angiogenic and proteolytic components in comparison with endometrium and peritoneal fluid from control women, suggesting that these systems play a role in the pathogenesis of endometriosis [18–21,23,24,53–55]. Peritoneal fluid is a dynamic media with continuous changes in the volume, the cellular components and cytokines. Moreover, it has been described that some inflammatory, immunological and oxidative stress components are dysregulated in endometriotic peritoneal fluid [56–60]. All of these alterations could dysregulate miRNA expression in stromal cells of endometrial fragments migrated to peritoneum, facilitating the implantation of ectopic lesions. In a previous report, we showed that endometrial-peritoneal interactions increased the expression of angiogenic and proteolytic factors in endometrial cells and suggested that this contributes to the

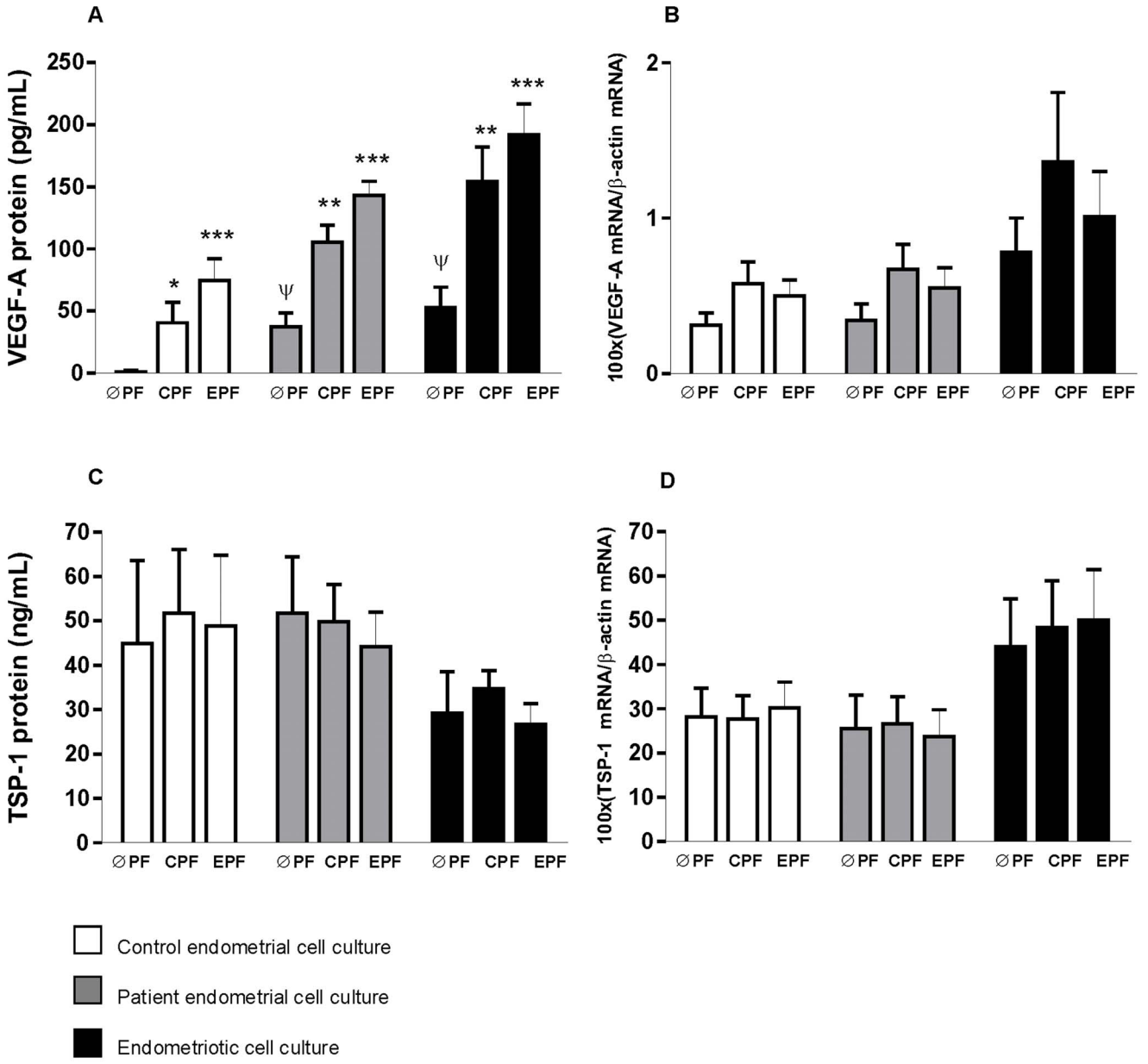

Figure 1. Peritoneal fluid (PF) effects on VEGF-A and TSP-1 expression in stromal cell cultures from control endometrial tissue, and patient endometrial and endometriotic tissues from women with endometriosis. ØPF, without PF; CPF, control PF; EPF, endometriotic PF. Data are expressed as Mean ± SEM. * $p<0.05$; ** $p<0.01$; *** $p<0.001$; vs ØPF from same tissue culture, ψ $p<0.05$ vs ØPF from control endometrium culture. A: VEGF-A protein; B: VEGF-A mRNA; C: TSP-1 protein; D: TSP-1 mRNA.
doi:10.1371/journal.pone.0062370.g001

establishment of endometriotic lesions [9]. However, the specific mechanisms by which peritoneal fluid components modulate the expression of angiogenic and proteolytic factors in endometrial and endometriotic cells have not been previously elucidated.

The present study shows that peritoneal fluid from women with endometriosis induces the highest decrease in angiogenesis-related miRNAs and the highest increase in VEGF-A protein levels in endometrial cell cultures from patients. The increase in protein levels without significant modification of mRNA levels could suggest a miRNA-mediated action on post-transcriptional regulation.

miRNAs, which have emerged as important regulators of gene expression, are involved in most cellular processes and many diseases, including endometriosis [44–46,61,62]. An increasing number of studies have described the relationship between miRNAs and angiogenesis [26–28,47] and emerging data suggest that dysregulation of miRNA expression is involved in endometriosis [32,35,43,45,46], increasing the likelihood that miRNAs could be used as biomarkers and therapeutic tools for this disease [38,63,64].

In the present study, treatment with peritoneal fluid resulted in decreased levels of miRNAs related to angiogenesis (miR-16, -17-5p, -20a, -125a, -221 and –222) and an increase in VEGF-A protein levels. The six miRNAs assessed in this study were selected for this *in vitro* model because they regulate VEGF-A expression directly or indirectly [28,29,44,65]. We found a significant

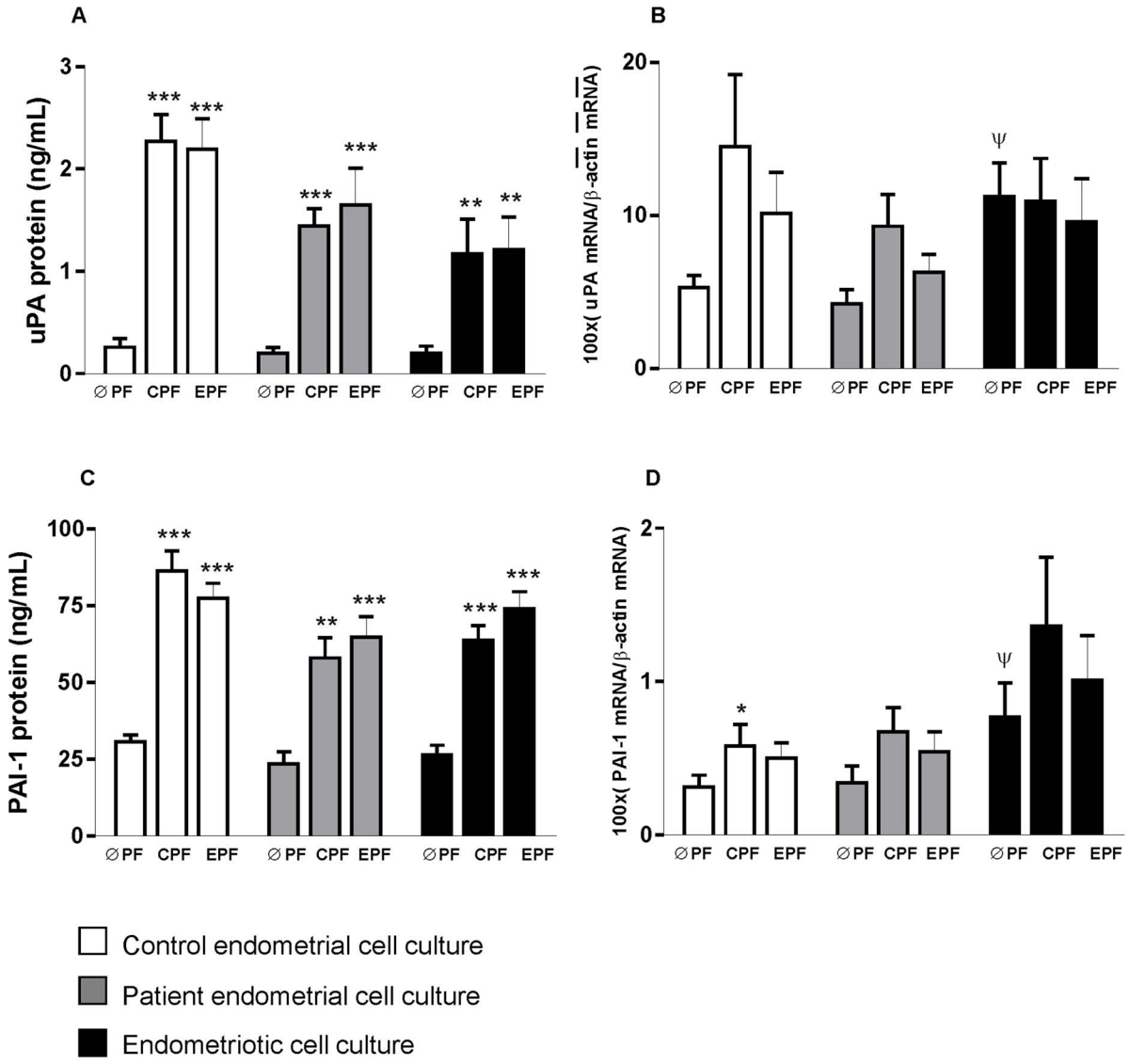

Figure 2. Peritoneal fluid (PF) effects on uPA and PAI-1 expression in stromal cell cultures from control endometrial tissue, and patient endometrial and endometriotic tissues from women with endometriosis. ØPF, without PF; CPF, control PF; EPF, endometriotic PF. Data are expressed as Mean ± SEM. * $p<0.05$; ** $p<0.01$; *** $p<0.001$; vs ØPF from same tissue culture, ψ $p<0.05$ vs ØPF from control endometrium culture. A: uPA protein; B: uPA mRNA; C: PAI-1 protein; D: PAI-1 mRNA.
doi:10.1371/journal.pone.0062370.g002

correlation between the decrease in miR-16 and the increase in VEGF-A in response to peritoneal fluid exposure in endometrial and endometriotic cell cultures. This correlation could indicate regulation of VEGF-A translation by miR-16. It has been shown that VEGF-A is a target gene for miR-16 in several cell types, indicating that miR-16 could be an important regulator of angiogenesis [30,47,46]. However, further experiments would be needed to test the hypothesis.

Furthermore, a significant inverse correlation was observed after peritoneal fluid treatment between the increase in VEGF-A protein expression and miR-17-5p, miR-20a, miR-125a and miR-222 levels in endometriotic cell cultures. These results suggest that peritoneal fluid modulates angiogenesis in endometrial and endometriotic stromal cells via miRNA action.

miRNAs -17-5p and -20a are contained in the miR-17-92 cluster, which has a complex role in angiogenesis [30,66,67]. While miR-17-5p, has pro-angiogenic activity [68,69], miR-20a displays anti-angiogenic activity by targeting VEGF-A [70]. Recently, Doebele *et al.* [66] showed that miR-17 and miR-20a exhibit a cell-intrinsic anti-angiogenic activity in endothelial cells. Another angiomiRs, miR-221 and miR-222, with a demonstrated anti-angiogenic activity by targeting c-kit, have been shown to inhibit endothelial cell migration, proliferation, and angiogenesis *in vitro* [71–73].

Several groups have studied peritoneal-endometrial interactions, showing that peritoneal fluid components (cytokines, growth factors, steroid hormones, and angiogenic and proteolytic factors) play an important role in the pathogenesis of endometriosis [13,56,57,74]. Moreover, cell types such as macrophages and endometrial and red blood cells have been detected in peritoneal fluid. Endometriotic peritoneal fluid reportedly induces the production and release of VEGF by neutrophils [9,12,54]. Our data show that endometriotic peritoneal fluid induced the highest increase in VEGF-A protein levels and the lowest miRNA expression in endometrial cell culture

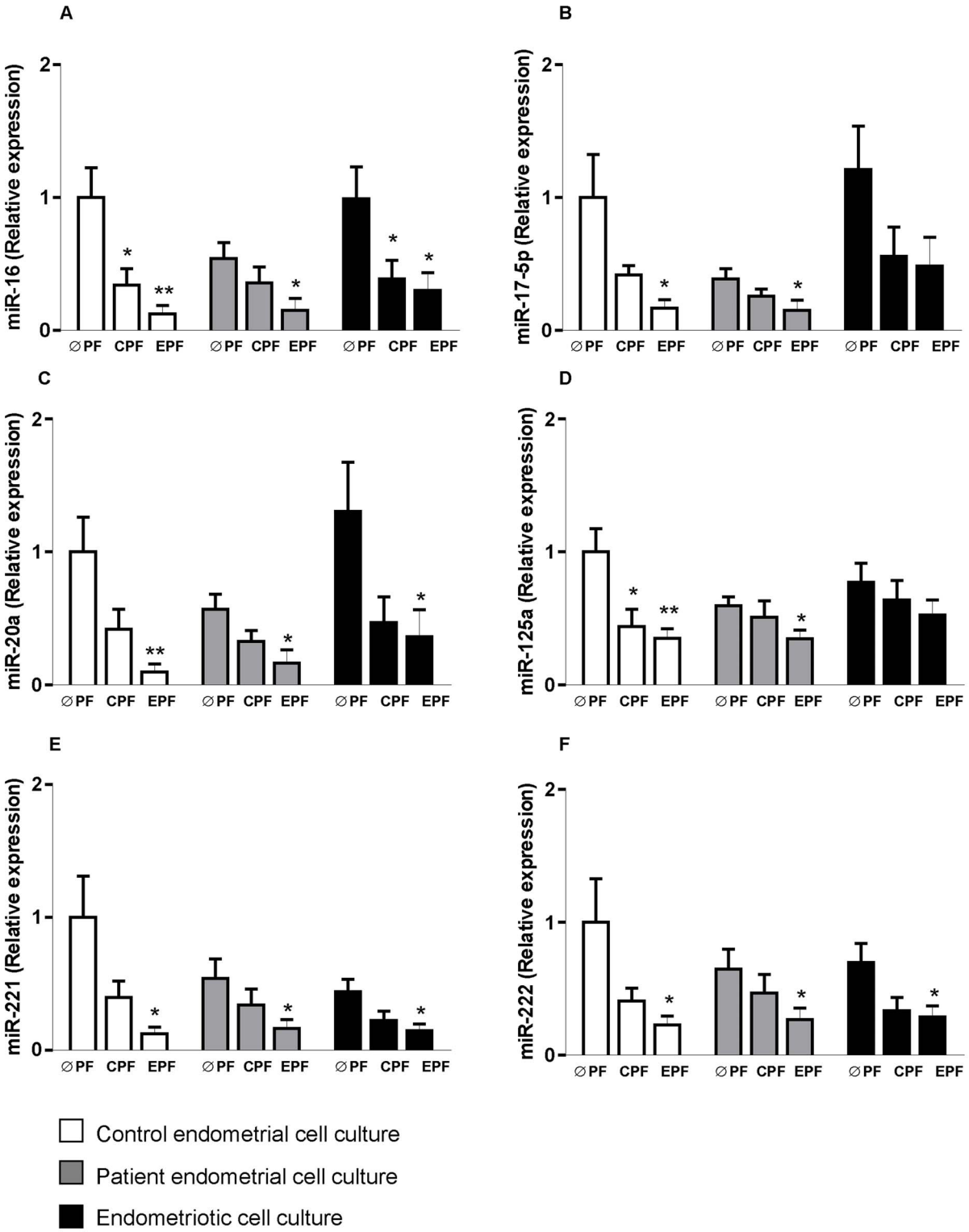

Figure 3. Peritoneal fluid (PF) effects on miRNA expression in stromal cell cultures from control endometrial tissue, and patient endometrial and endometriotic tissues from women with endometriosis. ØPF, without PF; CPF, control PF; EPF, endometriotic PF. *$p<0.05$; **$p<0.01$; $p<0.001$ vs ØPF same tissue culture. miRNA expression is presented as fold change relative to control stromal cell culture without peritoneal fluid (control endometrium ØPF = 1). A: miR-16; B: miR-17-5p; C: miR-20a; D: miR-125a; E: miR-221; F: miR-222.
doi:10.1371/journal.pone.0062370.g003

from women with endometriosis. Therefore, we hypothesized that an increase in cytokines or growth factors present in endometriotic peritoneal fluid contribute to the increase in angiogenic processes via a reduction of miRNAs that inhibit the translation of angiogenic factors. Hence, this reduction would induce an increase in protein levels of their target genes.

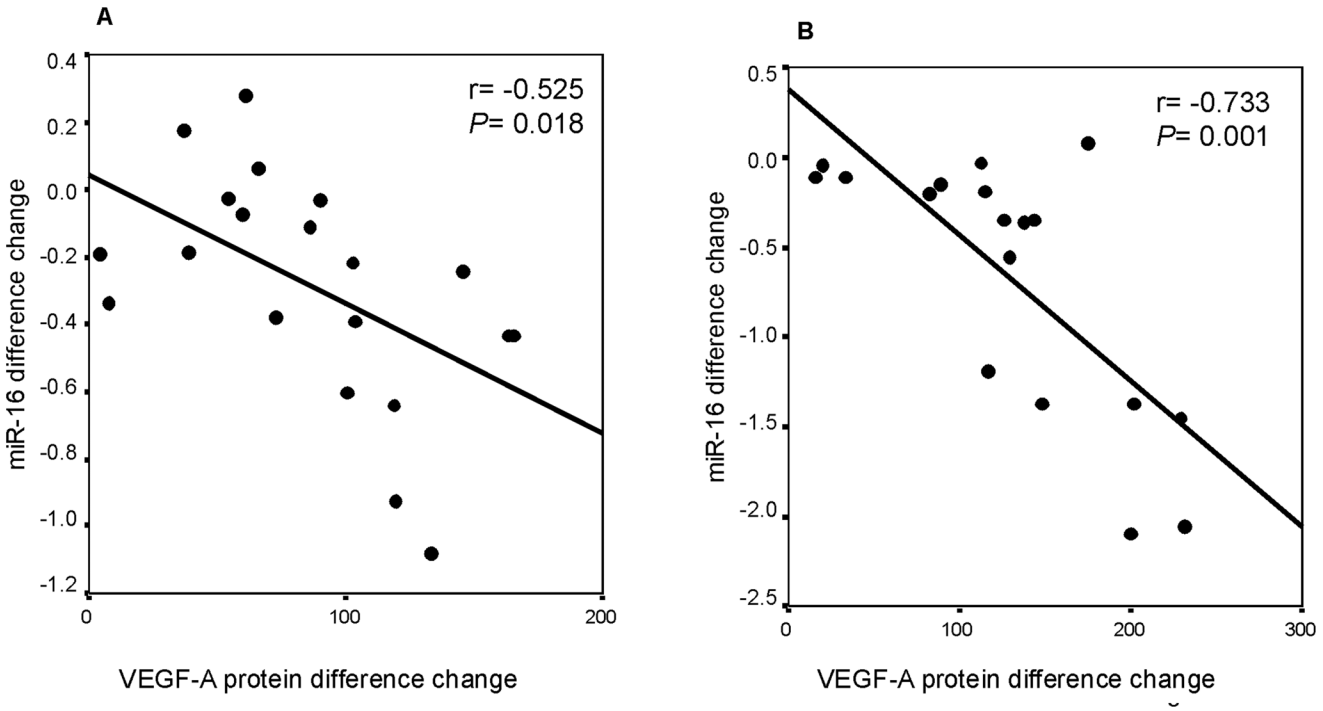

Figure 4. Correlation between changes in miR-16 vs. VEGF-A protein levels in eutopic endometrial (A) and endometriotic cell cultures (B) from women with endometriosis after treatment with both peritoneal fluid pools compared to cell cultures without peritoneal fluid treatment. Change: differences between parameter levels with and without peritoneal fluid treatment.
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Extracellular remodelling is necessary during the initial stages of angiogenesis. The enzymes involved in extracellular proteolysis include components of the plasminogen system [75,76]. Given that VEGF-A has been shown to induce uPA expression [75], it seemed reasonable to study angiogenic and fibrinolytic systems in our study subjects simultaneously. Furthermore, it has been reported that uPA levels are significantly higher in the endometrium from women with endometriosis than in controls [23,53,55,77–79], In the present study, we observed a significant induction of uPA and PAI-1 protein in the stromal cell culture in response to the presence of both peritoneal fluids. Moreover, a significant positive correlation was observed between the changes in VEGF-A and uPA protein levels in control endometrial culture after exposure to peritoneal fluid pools. The increase in proteolytic factors induced by peritoneal fluid from patients may also favour angiogenesis and invasive properties in this tissue.

The relationship between protein and mRNA levels of several angiogenic and proteolytic components may reflect the posttranscriptional regulation of the expression of these factors and their role in the development of endometriosis. It is important to emphasize that peritoneal fluid induced the expression of VEGF-A and uPA proteins but not of their corresponding mRNAs in the present study, which could be explained by miRNA action.

Limitations of the present study: In this study we have only evaluated six miRNAs, but it would be important to enlarge the number of angiomiRs to assess the importance of miRNAs in the regulation of the angiogenesis in the endometriosis development. Future functional studies (gain and loss of function experiments) are required to confirm the correlations between miRNAs and protein levels observed in the present study. Endometriosis is a multifactorial disease in which systems like angiogenesis, inflammation, immune system or epigenetics may be implicated in the pathogenesis of this disease. So, studying miRNAs action on establishment of ectopic lesions it is not enough to complain this complicated disease. It would be necessary to evaluate the implication of other systems in miRNAs regulation to better understand the cellular processes under the endometriosis development.

Conclusions

In this study we have observed that peritoneal fluid from women with endometriosis decreased the expression of six angiomiRs that could act as modulators of the translation of angiogenic and proteolytic factors in patient endometrial cells. Moreover, we have observed an inverse significative correlation between miRNA and protein levels. However, further validation of the targets of these differentially expressed miRNAs is necessary to clarify the role of miRNAs in the regulation of angiogenesis in endometriosis.

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Author Contributions

Conceived and designed the experiments: AB-B AE. Performed the experiments: AB-B LAR MC. Analyzed the data: AB-B JG-E LAR JG JM-A MC FE AE. Contributed reagents/materials/analysis tools: JG-E JG. Wrote the paper: AB-B JG-E LAR JG JM-A MC FE AE.

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human reproduction

ORIGINAL ARTICLE ***Gynaecology***

Peritoneal fluid modifies the microRNA expression profile in endometrial and endometriotic cells from women with endometriosis

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STUDY QUESTION: Could peritoneal fluid (PF) from patients with endometriosis alter the microRNA (miRNA) expression profile in endometrial and endometriotic cells from patients?

SUMMARY ANSWER: PF from patients with endometriosis modifies the miRNA expression profile in endometrial cells from patients.

WHAT IS KNOWN ALREADY: Angiogenesis is a pivotal system in the development of endometriosis, and dysregulated miRNA expression in this disease has been reported. However, to our knowledge, the effect of PF from patients on the miRNA expression profile of patient endometrial cells has not been reported. Moreover, an effect of three miRNAs (miR-16-5p, miR-29c-3p and miR-424-5p) on the regulation of vascular endothelial growth factor (VEGF)-A mRNA translation in endometrial cells from patients with endometriosis has not been demonstrated.

STUDY DESIGN, SIZE, DURATION: Primary cultures of stromal cells from endometrium from 8 control women (control cells) and 11 patients with endometriosis (eutopic cells) and ovarian endometriomas (ectopic cells) were treated with PF from control women (CPF) and patients (EPF) or not treated (0PF) in order to evaluate the effect of PF on miRNA expression in these cells.

PARTICIPANTS/MATERIALS, SETTING, METHODS: MiRNA expression arrays (Affymetrix platform) were prepared from cells (control, eutopic, ectopic) treated with CPF, EPF or 0PF. Results from arrays were validated by quantitative reverse transcription–polymerase chain reaction in cultures from 8 control endometrium, 11 eutopic endometrium and 11 ovarian endometriomas. Functional experiments were performed in primary cell cultures using mimics for miRNAs miR-16-5p, miR-29c-3p and miR-424-5p to assess their effect as VEGF-A expression regulators. To confirm a repressive action of miR-29c-3p through forming miRNA:*VEGFA* duplexes, we performed luciferase expression assays.

MAIN RESULTS AND THE ROLE OF CHANCE: EPF modified the miRNA expression profile in eutopic cells. A total of 267 miRNAs were modified in response to EPF compared with 0PF in eutopic cells. Nine miRNAs (miR-16-5p, miR-21-5p, miR-29c-3p, miR-106b-5p, miR-130a-5p, miR-149-5p, miR-185-5p, miR-195-5p, miR-424-5p) that were differently expressed in response to EPF, and which were potential targets involved in angiogenesis, proteolysis or endometriosis, were validated in further experiments (control = 8, eutopic = 11, ectopic = 11). Except for miR-149-5p, all validated miRNAs showed significantly lower levels (miR-16-5p, miR-106b-5p, miR-130a-5p; miR-195-5p and miR-424-5p, $P < 0.05$; miR-21-5p, miR-29c-3p and miR-185-5p, $P < 0.01$) after EPF treatment in primary cell cultures from eutopic endometrium from patients in comparison with 0PF. Transfection of stromal cells with mimics of miRNAs miR-16-5p, miR-29c-3p and miR-424-5p showed a significant down-regulation of VEGF-A protein expression. However, *VEGFA* mRNA expression after mimic transfection was not significantly modified, indicating the miRNAs inhibited VEGF-A mRNA translation rather than degrading *VEGFA* mRNA. Luciferase experiments also corroborated VEGF-A as a target gene of miR-29c-3p.

LIMITATIONS, REASONS FOR CAUTION: The study was performed in an *in vitro* model of endometriosis using stromal cells. This model is just a representation to try to elucidate the molecular mechanisms involved in the development of endometriosis. Further studies to identify the pathways involved in this miRNA expression modification in response to PF from patients are needed.

WIDER IMPLICATIONS OF THE FINDINGS: This is the first study describing a modified miRNA expression profile in eutopic cells from patients in response to PF from patients. These promising results improve the body of knowledge on endometriosis pathogenesis and could open up new therapeutic strategies for the treatment of endometriosis through the use of miRNAs.

STUDY FUNDING/COMPETING INTEREST(S): This work was supported by research grants by ISCIII and FEDER (PI11/00091, PI11/00566, PI14/01309, PI14/00253 and FI12/00012), RIC (RD12/0042/0029 and RD12/0042/0050), IIS La Fe 2011-211, Prometeo 2011/027 and Contrato Sara Borrell CD13/0005. There are no conflicts of interest to declare.

Key words: endometriosis / microRNA / peritoneal fluid / angiogenesis / VEGF-A

Introduction

Endometriosis is one of the most common gynecological diseases, whose prevalence is estimated at ~10% of women of reproductive age and up to 50% of infertile women (Burney and Giudice, 2012). It is characterized by the presence of endometrial tissue outside the uterus, and it is associated with pain and infertility (McKinnon *et al.*, 2012).

Nowadays, the most accepted theory explaining the development of endometriosis is the retrograde menstruation theory (Sampson, 1927). This theory proposes that endometrial fragments migrate to the peritoneum in a retrograde way during menstruation. In women with endometriosis, these endometrial fragments are able to survive, proliferate and develop new vessels to ensure the establishment of ectopic lesions. Although endometriosis is a benign disease, some features are in common with metastatic processes such as an aberrant angiogenesis. Hence, several groups have analyzed the important role of angiogenesis in the pathogenesis of endometriosis (Donnez *et al.*, 1998; Gilabert-Estellés *et al.*, 2007, 2012; Ramón *et al.*, 2011; Rahmioglu *et al.*, 2012; Rocha *et al.*, 2013; Braza-Boïls *et al.*, 2014).

Angiogenesis is a complex process regulated by a balance between promoters (proangiogenic factors) and inhibitors (antiangiogenic factors) and is essential for supplying oxygen and nutrition to tissues. Among these factors, the main regulator for angiogenesis is known to be the vascular endothelial growth factor (VEGF) and VEGF receptor (VEGFR) system, in which VEGF-A plays a pivotal role (Shibuya, 2008).

Ectopic lesions located in the pelvic peritoneum are immersed in peritoneal fluid (PF); therefore, the study of PF components and their effects on the development of endometriosis is a crucial objective to better understand this pathological condition (Cosín *et al.*, 2010; Berbic and Fraser, 2011; Braza-Boïls *et al.*, 2013; Olkowska-Truchanowicz *et al.*, 2013). In this context, increased levels of peritoneal macrophages and various proinflammatory and proangiogenic cytokines, abnormal T and B lymphocytes and VEGF-A have been reported in the PF from patients (Giudice and Kao, 2004; Gilabert-Estellés *et al.*, 2007; Martínez-Román *et al.*, 1997; Olkowska-Truchanowicz *et al.*, 2013; Rocha *et al.*, 2013). More recently, Berkes *et al.* (2014) described that 49% of studied patients with endometriosis presented neutrophil extracellular traps (NETs) in the PF, whereas control women rarely showed NET formation. They also observed that the highest percentage of NET-positive PFs was observed in patients with Stage I and II disease. These results suggest that NETs could play a role in initiation of the endometriosis.

MicroRNAs (miRNAs) are non-coding RNAs that may regulate angiogenesis through the modulation of RNA translation (Bartel, 2009). Several studies have reported the influence of miRNAs on the expression of proteins involved in physiological and pathological conditions (Burney *et al.*, 2009; Ohlsson-Teague *et al.*, 2009, 2010; Kuokkanen *et al.*, 2010; Ramón *et al.*, 2012). In relation to gynecological diseases, it has been suggested that altered expression of miRNAs may be involved in the development of endometriosis (Pan *et al.*, 2007, 2008; Toloubeydokhti *et al.*, 2008; Burney *et al.*, 2009; Guo, 2009; Ohlsson-Teague *et al.*, 2009, 2010; Hawkins *et al.*, 2011; Ramón *et al.*, 2011; Gilabert-Estellés *et al.*, 2012; Braza-Boïls *et al.*, 2013, 2014; Laudanski *et al.*, 2013). In a recent study (Braza-Boïls *et al.*, 2014), we described dysregulated miRNA expression in endometriosis, including changes in miR-16-5p, miR-29c-3p and miR-424-5p. *In silico* studies showed that these three miRNAs may regulate VEGF-A expression.

Several studies have indicated that miR-424-5p may be involved in angiogenesis regulation (Wang and Olson, 2009; Chamorro-Jorganes *et al.*, 2011).

Moreover, the role of miR-16-5p and miR-424-5p in the cell-intrinsic angiogenic activity of endothelial cells (ECs) has been investigated (Chamorro-Jorganes *et al.*, 2011), and the authors concluded that both miRNAs directly targeted VEGF-A. Therefore, these miRNAs could participate in the regulation of the angiogenic functions of ECs.

In relation to the role of miR-29c in angiogenesis, a previous study in rats confirmed that VEGF-A is a direct target of miR-29a,c specifically suppressing endogenous VEGF-A translation *in vitro* (Yang *et al.*, 2013).

To our knowledge, the direct effect of these three miRNAs (miR-16-5p, miR-29c-3p and miR-424-5p) on the regulation of VEGF-A translation in endometrial and endometriotic cells from patients with endometriosis has not been demonstrated.

In the present study, we investigated the role of PF from patients with endometriosis on the miRNA expression profile in primary cell cultures of stromal cells from control and eutopic endometrium and ovarian endometrioma from patients. Moreover, we evaluated the relationship of miRNAs to the aberrant angiogenesis observed in endometriosis.

Materials and Methods

Ethics statement

Written informed consent was obtained from all patients and controls, and the study was approved by the Ethical Committee from Hospital Universitario y Politécnico La Fe, Valencia, Spain (#2008/0111) and Hospital General Universitario, Valencia, Spain (#PBL00093).

Clinical groups

Patients

Caucasian women with moderate or severe endometriosis (Stages III and IV, revised American Society for Reproductive Medicine classification system, 1997) were studied. All women underwent laparoscopic surgical examination of the abdominal cavity and complete excision of endometriotic tissue. The presence of the disease was suspected either clinically or by ultrasonography and confirmed by surgical findings and post-operative pathological examination. Laparoscopic examination of the abdominal cavity excluded the presence of any other pelvic pathology that could potentially confound the data observed.

Controls

Normal endometrial tissues were obtained from fertile women without endometriosis who underwent surgery for tubal sterilization. The absence of endometriosis was confirmed by meticulous examination of the pelvic and extrapelvic peritoneum, ovaries, intestine and diaphragm in order to detect typical or atypical endometriotic lesions.

PF from controls and patients were centrifuged at 1500 × *g* for 30 min at 4°C, filtered through a 0.2 μm pore size membrane, and stored at −80°C.

Women affected by menorrhagia or hypermenorrhea or women who had been pregnant or breastfeeding during the previous 6 months were excluded from the study. None of the women had received any form of hormone therapy for at least 3 months before the study.

Tissue samples and cell lines

In order to isolate stromal cells, 11 endometrial tissues (eutopic cells) (mean age 32 years; range 19–40) and 11 ovarian endometriomas (ectopic cells) from women with moderate or severe endometriosis (Stages III and IV) (mean age 30 years; range 19–42) and control endometrial tissue (control cells) from 8 women without the disease (mean age 36 years; range 24–43) were obtained.

The EC line EA.hy926 was obtained from the American Type Culture Collection (Manassas, VA, USA). ECs were maintained in phenol-red free Dulbecco's modified Eagle's medium supplemented with 2 mM glutamine and 10% fetal bovine serum (Life Technologies, Madrid, Spain). A human colon cancer cell line HCT-116 deficient for Dicer (HCT-DK) was a kind gift from Dr Renato Baserga (Thomas Jefferson University, PA, USA). HCT-DK were cultured in McCoy's 5A (Sigma-Aldrich, Madrid, Spain) supplemented with 2 mM glutamine and 10% fetal bovine serum.

PF pools

PF pools consisted of 10 PFs from women with endometriosis (endometriotic PF pool, EPF) (mean age 33 years; range 27–39) and 10 PFs from fertile women without endometriosis (control PF pool, CPF) (mean age 37 years; range 21–47) in the proliferative phase of the menstrual cycle.

Primary cell culture of stromal cells from endometrial and endometriotic tissues and PF exposure

Cell culture and exposure to PF pools were performed as previously described (Braza-Boïls *et al.*, 2013), and functional experiments were performed in cultures at passage 2–4. The cell lines EA.hy926 and HCT-DK were cultured according to the American Type Culture Collection protocols.

Cell transfections

Cells were seeded 24 h before transfection in complete medium without antibiotics and transfected with 100 nM of chemically modified double-stranded RNAs that mimic endogenous miRNAs (miR-16-5p, miR-29c-3p, miR-424-5p or scrambled control) by using the siPORT™ *NeoFX*™ transfection agent from Life Technologies in OPTIMEM according to the manufacturer's instructions. After 24 h, cells were collected for subsequent mRNA and protein analyses. All transfections were performed in triplicate.

RNA extraction

Total RNA from cells stimulated with EPF or CPF and without stimulation (0PF) was extracted using mirVana miRNA isolation kit (Life Technologies), according to the manufacturer's protocol. Total RNA from transfected cell cultures was isolated using Trizol Reagent (Life Technologies). Yield and purity of RNA were measured using a NanoDrop ND-1000 spectrophotometer (Nanodrop Products, Wilmington, DE, USA), and the RNA integrity was analyzed using the Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA). All samples employed for microarray assays presented a RNA integrity number $\geq$9.0.

Analysis of miRNA expression profiles

MiRNA expression profiles were studied in primary cell cultures of stromal cells from four eutopic endometrium, three ovarian endometrioma and three control endometrium exposed to EPF, CPF or 0PF. Affymetrix platform, GeneChip miRNA 2.0 arrays (Affymetrix, Santa Clara, CA, USA) were employed according to the manufacturer's protocol. Arrays were prepared in our Array Facility (IIS La Fe, Valencia, Spain). Data analysis was performed employing PARTEK Genomic Suite software (PARTEK, Inc., St Louis, MO, USA) and normalized using the robust multiarray analysis (RMA) algorithm. Analysis of variance (ANOVA) statistical analysis allowed us to generate a list of differently expressed miRNAs, with significance set at a P-value < 0.05.

Validation of selected mature miRNAs by quantitative real-time RT–PCR

Target genes of differentially expressed miRNAs in response to PF exposure were assessed using the following miRNA binding sites prediction programs: miRBase (http://microrna.sanger.ac.uk/) (Kozomara and Griffiths-Jones, 2011), miRSVR (http://www.microrna.org) (Betel *et al.*, 2010), TargetScan (http://www.targetscan.org) (Lewis *et al.*, 2005) and DIANA-microT (http://diana.imis.athena-innovation.gr) (Paraskevopoulou *et al.*, 2013). Nine miRNAs (miR-16-5p, miR-21-5p, miR-29c-3p, miR-106b-5p, miR-130a-5p, miR-185-5p, miR-195-5p, miR-424-5p) with potential targets involved in angiogenesis, proteolysis or endometriosis were selected to be validated by *quantitative real-time reverse transcription–polymerase chain reaction* (qRT–PCR) in a larger number of experiments, including the cell cultures in which microarray experiments were performed. RNA RNU6B was employed as endogenous control.

Mature miRNAs quantification was performed by miRCURY LNA™ Universal RT microRNA PCR (Exiqon, Vedbaek, Denmark) employing a Light cycler 480 II instrument (Roche Applied Science, Penzberg, Germany).

VEGF-A protein quantification

VEGF-A protein levels from supernatants were measured using a commercially available enzyme-linked immunosorbent assay (ELISA) (Human VEGF, IBL International, Hamburg, Germany). No cross-reactivity or interference with platelet-derived growth factor was observed. This assay recognizes human VEGF-A_{165} and VEGF-A_{121} isoforms. The intra-assay and inter-assay variation coefficients were 4–6% and 7–10%, respectively.

VEGF-A protein expression from cells was quantified by western blot (anti-VEGF antibody ab46154, Abcam, Cambridge, UK), which recognizes both human VEGF-A_{165} and VEGF-A_{121} isoforms.

VEGF-A mRNA

VEGF-A mRNA was quantified by qRT–PCR. Briefly, RNA (400 ng) and SuperScript™ III First-Strand Synthesis System (Life Technologies) were used for reverse transcription reactions. *VEGFA* and beta-actin (*ACTB*) (as endogenous reference control) gene expression was quantified by polymerase chain reaction (PCR) (probe references: Hs00900055_m1 and Hs99999903_m1, respectively, from Life Technologies). The $2^{-\Delta Ct}$ method was followed to calculate the relative abundance of mRNA compared with endogenous control expression (Ct = threshold cycle; ΔCt = Ct endogenous control—Ct sample gene).

Luciferase experiments

Plasmid construction

To confirm the repressive action of miR-29c-3p through forming miRNA:-*VEGFA* duplexes, we inserted a fragment of the *VEGFA* 3′ untranslated region (UTR) containing the binding site for this miRNA into a luciferase expression vector, generating the luciferase reporter construct pMIR-VEGFA-3′UTR. Briefly, pMIR-*VEGFA*-3′UTR contained a fragment located at nt +1575-1829 of the *VEGFA* 3′UTR. The PCR fragment was cloned into the pCR 2.1 vector (Life Technologies™). Positive clones were digested with SacI and HindIII (New England Biolabs, Ipswich, MA, USA), and the insert was subcloned into the luciferase reporter plasmid pMIR-REPORT™ (Life Technologies) previously digested with SacI and HindIII. Insertion of the *VEGFA* 3′UTR fragment was checked by sequencing (ABI3130 XL, Life Technologies Corporation, Carlsbad, CA, USA). All sequence analyses and alignments were performed with the SeqmanPro program (Lasergene version 7.1, DNASTAR, Madison, WI, USA).

To generate mutations in the predicted target site for the miR-29c-3p, seven nucleotides located in the seed sequence were deleted using the QuikChange site-directed mutagenesis kit (Agilent Technologies). Sequencing was performed to check for the deletion of the seed sequences. The primers used for cloning and mutagenesis are detailed in Supplementary data, Table SI.

Luciferase vector transfection

MiR-29c-3p mimic was co-transfected with pMIR-*VEGFA*-3′UTR and *Renilla* vector pRL-TK (Promega, Madison, WI, USA) into the HCT-DK cell line. Cells were seeded at a density of 80 000 cells/well in 24-well plates with McCoy's 5A supplemented with 10% fetal calf serum without antibiotics. The following day, cells were co-transfected with scrambled precursor (SCR) or miR-29c-3p mimic (both pMIR-REPORT plasmids—1000 ng/well—wild type or mutated for the miRNA seed site) and 100 ng/well of *Renilla* luciferase control plasmid (pRL-TK, Promega) using Lipofectamine LTX (Life Technologies), according to the manufacturer's instructions. Luciferase assays were performed as previously described (Salloum-Asfar *et al.*, 2014). The enzymatic activities of *Renilla* and firefly luciferases were quantified in a Synergy 2 luminometer (Biotek, Winooski, VT, USA). Each combination of pMIR-REPORT (wild-type and mutated 3′UTR) and pRL-TK was tested in triplicate in five independent experiments. Firefly luciferase activity was normalized to *Renilla* luciferase activity for each transfected well. The normalized data were expressed as changes relative to the data of the cells transfected with 100 nM SCR mimic. SCR was taken as 100%.

Statistical analysis

Results from arrays were analyzed using PARTEK Genomic Suite Software. Comparisons between groups for all other analyses were performed by an unpaired *t*-test. Statistical tests were performed using the Statistical Package for the Social Sciences Release 20 for Windows (SPSS, Inc., Chicago, IL, USA)

Luciferase activity levels were compared between SCR and mimics in wild-type and mutant vectors using linear mixed models. Independent experiments were regarded as a random effects variable in the model, and SCR/miR and WT/MUT factors were regarded as fixed effects. Error bars were used to display SEs, and *P*-values < 0.05 were considered statistically significant. These analyses were performed using R software (version 3.0.2) (r-project.org).

Results

MiRNA expression profiles (Affymetrix platform)

The GeneChip miRNA 2.0 Array contains 1105 human probes for mature miRNAs and 1121 probes for their respective pre-miRNAs.

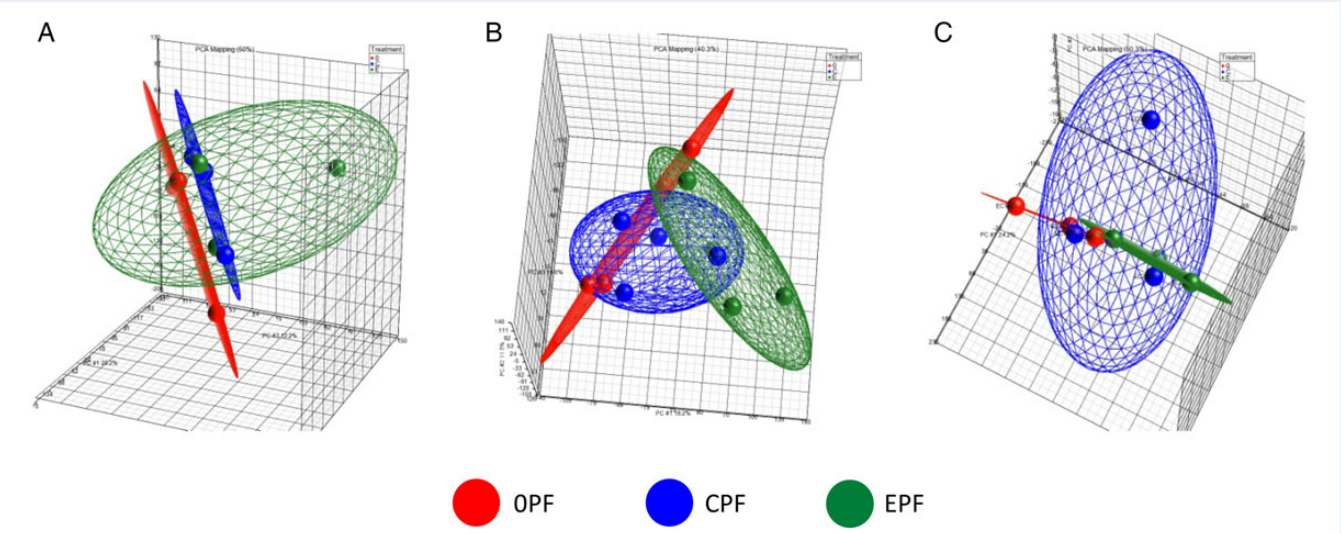

Figure 1 Principal component analysis performed from miRNA expression arrays (Affymetrix): (**A**) control cells, (**B**) eutopic cells and (**C**) ectopic cells. 0PF: without any treatment; CPF: treated with peritoneal fluid from control women and EPF: treated with peritoneal fluid from patients.

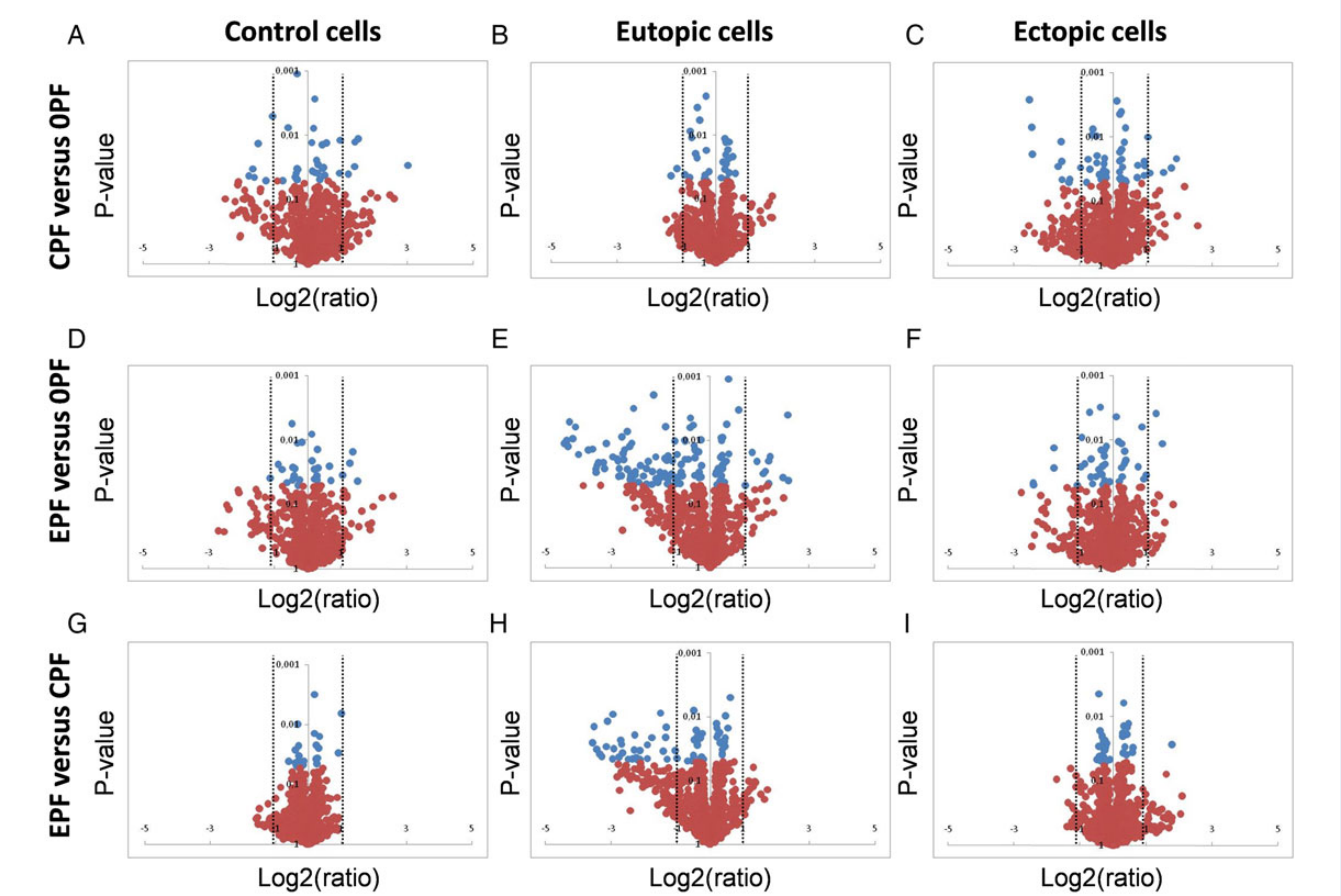

Figure 2 Volcano plots representing miRNA differently expressed in control, eutopic or ectopic cells in response to different treatments.

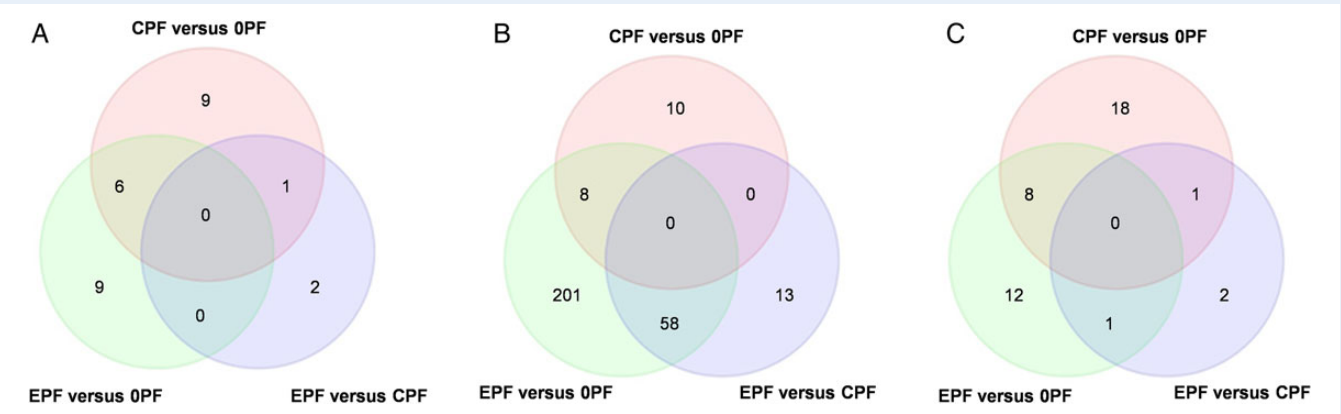

Figure 3 Venn diagrams representing the number of miRNAs dysregulated in each experimental condition: (**A**) control cells, (**B**) eutopic cells and (**C**) ectopic cells. Student's *t*-test.

Profiling of these RNAs was completed for three cultures from control endometrium (control cells), four from endometrium from patients (eutopic cells) and three from ovarian endometriomas (ectopic cells) treated with PFs from patients (EPF), controls (CPF) and without treatment (0PF). Principal component analysis revealed that control cells treated with CPF showed no modification in the miRNA expression pattern in comparison with untreated cells. Nevertheless, the miRNA expression was different in response to EPF (Fig. 1A). In contrast to control cells, eutopic and ectopic cells responded to EPF and CPF in a different way in terms of miRNA expression (Fig. 1B and C). Volcano plots from ANOVA test (Fig. 2) revealed that the major difference in miRNA expression was observed in eutopic cells after EPF treatment

Table I miRNA microarray expression and targets of miRNA selected for the PCR experiments.

miRNA (v. 15)[a]	miRNA (v. 20)[b]	miRNA sequence 5′–3′	Eutopic cells response to EPF compared with 0PF		Target
			Fold change	*P*-value *	
miR-16	**miR-16–5p**	UAGCAGCACGUAAAUAUUGGCG	−9.96870	0.04321	VEGFA, EGFR2, BCL2, FGFR1, COX2
miR-21	**miR-21-5p**	UAGCUUAUCAGACUGAUGUUGA	−16.96419	0.00620	TIMP3, TGFb2, SERPINB5, VEGFA, BCL2, EGFR, MMP2, HIF1a, MMP8, TGFb, TGFBR1, THBS1, TNFRSF11B
miR-29c	**miR-29c-3p**	UAGCACCAUUUGAAAUCGGUUA	−2.38095	0.01301	VEGFA, PDGFB-C, THSD4 (TSP-1D4), SERBP1, ADAMTS2, 5–7, 9, 17–19
miR-106b	**miR-106b-5p**	UAAAGUGCUGACAGUGCAGAU	−20.34633	0.01002	TGFBR2, MMP2, THSD3, CCNG2, ADAM9, IL8, MMP24, COL4A3, CCND1, TIMP2, CCND2, COL19A, FGF4, VEGFA
miR-130a	**miR-130a-3p**	CAGUGCAAUGUUAAAAGGGCAU	−19.25180	0.00517	SERPINE1, COL4A1, IL6R, COL4A5, VEGFA, COL1A2, SERPINB7, FAS (TNFR superfamily)
miR-149	**miR-149-5p**	UCUGGCUCCGUGUCUUCACUCCC	3.43946	0.04766	GPC1, FGFR1[c], EDNRA, TNFRSF19
miR-185	**miR-185-5p**	UGGAGAGAAAGGCAGUUCCUGA	−19.84907	0.01248	VEGFA, THSD7A, CLDN11, IL17R, HIF3a, EDA2R
miR-195	**miR-195-5p**	UAGCAGCACAGAAAUAUUGGC	−4.99004	0.00318	COL12A1, CDCA4, BCL2L2, VEGFA, CLDN12, CCND1, SERBP1, DICER1, ADAMTS5, GHR, CLDN2, ESRRA, ESRRG, ADAMTS1
miR-424	**miR-424-5p**	CAGCAGCAAUUCAUGUUUUGAA	−2.03838	0.04712	VEGFA, IL1, FGF2

ADAMTS2, 5–7, 9, 17–19: ADAM metallopeptidase with thrombospondin type 1 motif, 2, 5–7, 9, 17–19; BCL2: B-cell lymphoma 2; BCL2L2: BCL2-like 2; CCND1: cyclin D1; CCND2: cyclin D2; CCNG2: cyclin G2; CDCA4: cell division cycle associated 4; CLDN11: claudin 11; CLDN12: claudin 12; COL1A2: collagen, type I, alpha 2; COL4A1: collagen, type IV, alpha 1;COL4A3: collagen, type IV, alpha 3; COL4A5: collagen, type IV, alpha 5; COL12A1: collagen, type XII, alpha 1; COL19A: collagen, type IXX, alpha; COX2: cyclooxygenase 2; DICER1: dicer 1, ribonuclease type III; EDA2R: ectodysplasin A2 receptor; EDNRA: endothelin receptor type A; EGFR2: epidermal growth factor receptor 2; ESRRA: estrogen-related receptor alpha; ESRRG: estrogen-related receptor gamma; FAS (TNFR superfamily): Fas cell surface death receptor; FGF2: fibroblast growth factor 2; FGF4: fibroblast growth factor 4; FGFR1: fibroblast growth factor receptor 1; GHR: growth hormone receptor; GPC1: glypican 1; HIF1a-3a: hipoxia inducible factor 1–3, alpha subunit; IL1: interleukin 1; IL6R: interleukin 6 receptor; IL8: interleukin 8; IL17R: interleukin 17 receptor; MMP2: matrix metalloproteinase-2; MMP8: matrix metalloproteinase-8; MMP24: matrix metalloproteinase-24; PDGFB-C: platelet-derived growth factor polypeptide-C; SERPINE1: plasminogen activator inhibitor type 1; SERPINB5-7: serpin peptidase inhibitor, clade B, member 5–7; TGFb2: transforming growth factor beta 2; TGFBR1: transforming growth factor, beta receptor 1; TGFBR2: transforming growth factor, beta receptor 2; THBS1: thrombospondin 1; THSD3: thrombospondin, type 1, domain containing 3; THSD4: thrombospondin, type 1, domain containing 4; THSD7A: thrombospondin, type I, domain containing 7A; TIMP3-2: tissue inhibitor of metalloproteinases-3-2; TNFRSF11B: tumor necrosis factor receptor superfamily, member 11b; TNFRSF19: tumor necrosis factor receptor superfamily, member 19; VEGFA: vascular endothelial growth factor.

EPF, endometriotic peritoneal fluid; 0PF, without peritoneal fluid.

[a]Referred to miRBase database release (version 15).

[b]Referred to miRBase database release (version 20). MiRNAs are named in microarray according to miRBase version 16. However, the current classification is referred to miRBase 20 release.

[c]Chamorro-Jorganes *et al.* (2014).

*ANOVA.

(Fig. 2E and H). Moreover, it should be underlined that the majority of these miRNAs were down-regulated in response to EPF. The comparison between the response to EPF and to 0PF showed that eutopic cells presented the highest number of miRNAs significantly dysregulated: $P < 0.05$ and $\pm$2-fold change (Fig. 2H). Venn diagrams (Fig. 3) representing all of the differentially expressed ($P < 0.05$) human miRNA probes in the array showed that EPF modified the expression of some miRNAs >12-fold in eutopic cells compared with the other cultures. Among the 267 miRNAs that are modified in response to EPF compared with 0PF in eutopic cells (Fig. 3B), 82 corresponded to mature miRNAs (72 down-regulated and 10 up-regulated) ($P < 0.05$; $\pm$2-fold change) (Supplementary data, Table SII).

After the *in silico* study of the target genes for those miRNAs differentially expressed in eutopic cells from patients treated with EPF, we selected nine miRNAs related to angiogenesis (miR-16-5p, miR-21-5p, miR-29c-3p, miR-106b-5p, miR-130a-5p, miR-149-5p, miR-185-5p, miR-195-5p, miR-424-5p) for validation by qRT–PCR in a larger number of experiments. Eight of these miRNAs were down-regulated in the expression arrays (miR-16-5p, miR-21-5p, miR-29c-3p, miR-106b-5p, miR-130a-5p, miR-185-5p, miR-195-5p, miR-424-5p), and miR-149-5p was up-regulated. (Table I).

Validation by qRT–PCR

With the exception of miRNA-149-5p, which did not show increased levels, as the arrays results revealed (Fig. 4F), the other eight miRNAs showed statistically significant lower levels after EPF treatment in primary cell cultures from eutopic endometrium from patients (Fig. 4A–E, H and I). MiR-16-5p and miR-424-5p showed lower levels after CPF and EPF treatments in control cells. Ectopic cells reduced the expression of miR-16-5p, miR-29c-3p, miR-106b-5p, miR-130a-5p and miR-185-5p in the presence of both PF pools, but only the change in miR-16-5p was statistically significant.

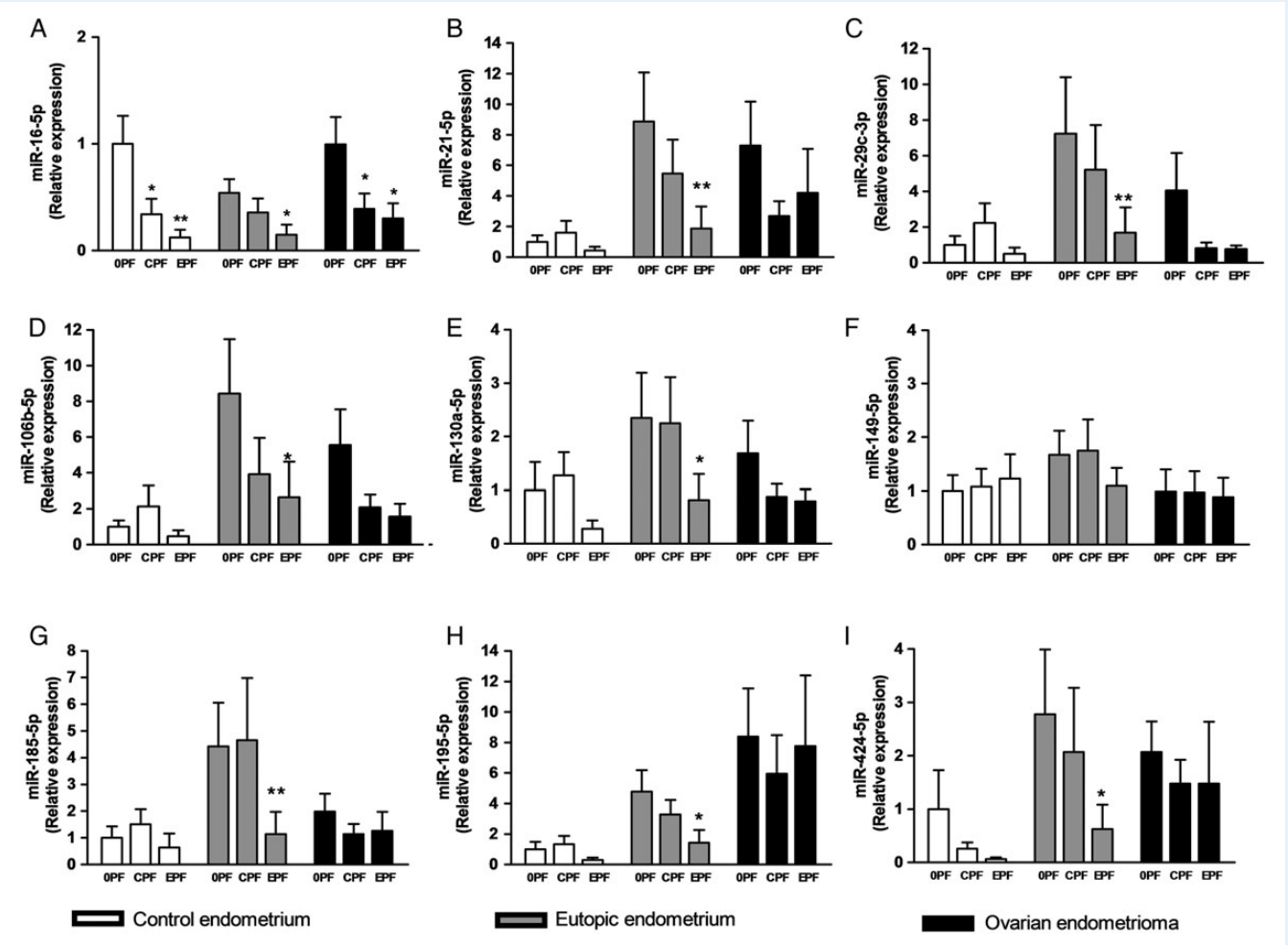

Figure 4 Nine miRNAs were selected from array results in order to be validated by qRT–PCR in control ($n = 8$), eutopic ($n = 11$) and ectopic ($n = 11$) cells treated with: ANOVA test *$P < 0.05$ and **$P < 0.01$.

Functional experiments using mimics

MiR-16-5p, miR-29c-3p and miR-424-5p were significantly down-regulated in eutopic cells after EPF treatment in the array validation phase. Recently, our group published a dysregulated miRNA expression profile in different lesions characteristic of endometriosis, including miR-16-5p, miR-29c-3p and miR-424-5p (Braza-Boïls *et al.*, 2014). Moreover, *in silico* analysis revealed that all three miRNAs could regulate VEGF-A translation (Table I).

To specifically investigate whether VEGF-A expression could be regulated by these three miRNAs, we transfected the EA.hy926 EC line and primary cell cultures from control and patient endometrium with mimics of miR-16-5p, miR-29c-3p and miR-424-5p (Fig. 5).

In order to assess the possible effect of endogenous miRNAs on mimic transfections, miRNAs levels were quantified by qRT–PCR (Supplementary data, Fig. S1). Results validated the effect of exogenous synthetic miRNAs used in the functional studies.

In the EA.hy926 cell line, transfection with miR-16-5p, miR-29c-3p or miR-424-5p mimics induced a reduction in VEGF-A expression versus scrambled mimic of 63 ± 11%, 76 ± 0.9% and 79 ± 0.9% ($P < 0.01$), respectively (Fig. 5A and D). When the same transfections with miR-16-5p, miR-29c-3p or miR-424-5p mimics were performed in primary cell cultures from controls and patient endometrium, VEGF-A expression was reduced versus scrambled mimic by 79 ± 20% ($P = 0.12$), 90 ± 0.2% and 90 ± 0.2% ($P < 0.001$) in endometrial cells from women without the disease (Fig. 5B and D) and 96% ($P < 0.001$), 79% and 78% ($P < 0.01$) in patient endometrial cells, respectively (Fig. 5C and D). In control cell cultures, different doses of mimics (20, 50 and 100 nM) were transfected. *VEGFA* mRNA levels were quantified after transfections, observing no statistically significant modifications in any of the studied cell types (Fig. 5E). Moreover, quantification of VEGF-A protein levels by ELISA showed that mimics seem to act in a dose-dependent manner (Supplementary data, Fig. S2).

Validation of miRNA-VEGF-A interaction

To test the hypothesis that miR-29c-3p can directly modulate VEGF-A expression, *VEGFA* 3′UTR was cloned downstream from the firefly luciferase open reading frame. Either the wild-type reporter construct or the miR-29c-3p binding site deleted construct were co-transfected in different experiments (Fig. 5G) in the HCT116-Dicer KO cell line, with a SCR or a miR-29c-3p mimic.

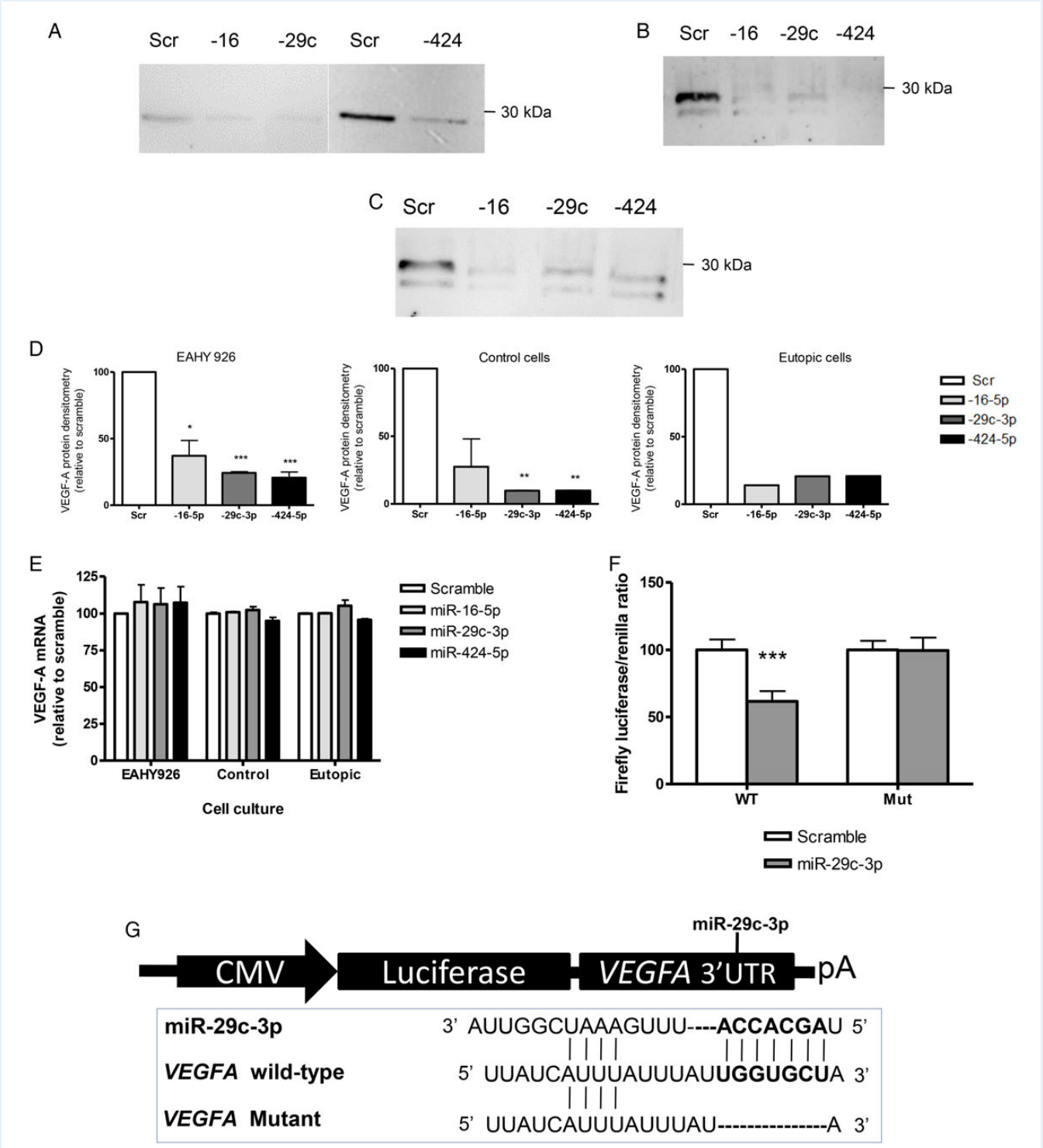

Figure 5 *In vitro* studies. (**A–C**) Representative western blots for VEGF-A after transfection of cells (A: EA.hy 926, B: control cells, C: eutopic cells) with scramble or miR-16-5p, miR-29c-3p and miR-424-5p mimics (100 nM) for 48 h. (**D**) Densitometric analysis of VEGF-A extracellular expression. (**E**) VEGF-A protein levels after control cell transfection with mimics (20, 50 and 100 nM) measured by ELISA. (**F** and **G**) Luciferase assays. (**F**) VEGF-A mRNA levels after mimic transfection (100 nM). (**G**) Schematic representation of miR-29c-3p predicted target site in *VEGFA* 3′ UTR. Complementarities between the seed region (seven nucleotides) of miR-29c-3p and 3′ UTR of *VEGFA* mRNA target site are shown. HCT116—The dicer KO cell line was co-transfected with scramble or miR-29c-3p mimic and pMIR-*VEGFA*-3′ UTR wild-type (WT) or mutated (Mut). All experiments were performed in triplicate ($n = 3$). ANOVA test $^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$.

Our results showed that the relative luciferase activity was significantly decreased in cells co-transfected with the wild-type construct and miR-29c-3p (62 ± 8%, $P < 0.001$). However, this inhibition was not observed when co-transfection was performed with the vector containing the specific mutated 3′UTR of *VEGFA* (Fig. 5F), indicating that *VEGFA* 3′UTR could be a direct target of miR-29c-3p.

Discussion

In the present study, we observed that PF from patients modified the miRNA expression profile in endometrial stromal cells from women with endometriosis, including miRNAs involved in angiogenesis. In a previous report (Braza-Boïls *et al.*, 2014), we described a dysregulated miRNA expression profile in endometrial and endometriotic tissues, including miR-16-5p, miR-29c-3p and miR-424-5p, and *in silico* studies showed that these three miRNAs may regulate VEGF-A expression. In the present study, we have performed functional studies employing mimics for these miRNAs, indicating that these miRNAs regulate VEGF-A translation not only in the EA.hy926 cell line but also in cells from endometrial tissues from women with and without endometriosis.

Angiogenesis plays an important role in multiple physiological and pathological processes including gynaecological diseases like endometriosis. Several miRNAs can control the expression of VEGF-A. MiR-29c-3p is a multifunctional miRNA implicated in several processes, including extracellular remodeling and angiogenesis, and can contribute to the formation of endometriotic lesions in patients with endometriosis (Braza-Boïls *et al.*, 2014). A study performed in rats (Yang *et al.*, 2013) demonstrated that VEGF-A is a direct target of miR-29a and miR-29c and these miRNAs suppressed endogenous VEGF-A expression *in vitro*. In the present study, we have observed that the transfection of miR-29c-3p in endometrial and endometriotic cells from patients with endometriosis significantly decreased VEGF-A protein expression. Furthermore, luciferase experiments indicated that VEGF-A is a direct target of miR-29c-3p also in humans.

Both miR-16-5p and miR-424-5p target the same 'seed sequence', the nucleotide sequence in which these miRNAs can bind to *VEGFA* mRNA, which implies that both miRNAs can share most of their target genes. In the present work, we observed a significant reduction in VEGF-A protein expression in primary cell cultures from controls and patients endometrium after transfection with miR-16-5p or miR-424-5p mimics. However, *VEGFA* mRNA expression after mimic transfection was not significantly modified. The decrease in protein levels without significant modification of mRNA levels indicates that these miRNAs mainly inhibit VEGF-A translation without degrading *VEGFA* mRNA, as has been described for several proteins (Braza-Boïls *et al.*, 2013). Indeed, in a previous study (Braza-Boïls *et al.*, 2013), we investigated the influence of PF from women with and without endometriosis on the expression of six miRNAs, including miR-16-5p, that modulate angiogenesis, as well as several angiogenic and proteolytic factors in endometrial and endometriotic cell cultures. We found a significant correlation between the decrease in miR-16-5p and the increase in VEGF-A protein, but not mRNA, in response to PF exposure in endometrial and endometriotic cell cultures.

In a previous report, Chamorro-Jorganes *et al.* (2011) investigated the role of miR-16-5p and miR-424-5p in the angiogenic activity of ECs and showed that both miRNAs directly targeted *VEGFA*. These results are in agreement with results obtained in the present report, in which we have observed that miR-16-5p and miR-424-5p can regulate VEGF-A protein levels in endometrial and endometriotic cells.

In a previous study (Braza-Boïls *et al.*, 2014), we suggested that miR-424-5p contributed, at least in part, to the higher VEGF-A levels observed in the endometrium from patients with endometriosis. Other authors indicated that miR-424-5p targets VEGF-A and plays an important role in down-regulating the angiogenic activity of this protein (Wang and Olson, 2009; Chamorro-Jorganes *et al.*, 2011). Moreover, Nakashima *et al.* (2010) reported that down-regulation of mir-424 can contribute to the abnormal angiogenesis in senile hemangioma.

MiRNAs may mediate cell-to-cell communication via exosomes (Boon and Vickers, 2013; Kosaka *et al.*, 2013). However, the mechanisms whereby miRNAs are packaged in exosomes and the selection of miRNAs secreted in each cell state are unclear. Exosomal miRNAs have been characterized in blood, urine and other body fluids, and exosomes can reflect their tissue or cell of origin by the presence of specific surface proteins (Zhang *et al.*, 2015). Moreover, a cell-phenotype modulation induced by miRNAs-enriched exosomes has been described (Hulsmans and Holvoet, 2013; Raposo and Stoorvogel, 2013; Rayner and Hennessy, 2013).

In order to perform miRNA-based communication, three steps are required. In a first step, miRNAs must be secreted from donor cells into exosomes. Second, miRNAs migrate into RNAse-protected vesicles in the recipient cell. And, finally, miRNAs must recognize their mRNA target and repress its translation (Boon and Vickers, 2013).

Although peritoneal macrophages are able to secrete miRNA-rich exosomes (Hulsmans and Holvoet, 2013) and could contribute to the final endometriotic phenotype by means of the aforementioned mechanism, our results showed that PF from patients modified miRNA expression in eutopic cells from patients with endometriosis, indicating not only that peritoneal factors could be involved in the endometriosis pathogenesis, but also that endometrial factors seem to be implicated. However, more studies are required in order to elucidate the mechanisms by which PF from patients is able to modify the receptor cell phenotype.

In conclusion, PF from patients modified the miRNA expression profile in endometrial cells from women with endometriosis. Functional studies employing mimics for miR-16-5p, miR-29c-3p and miR-424-5p suggested that these miRNAs regulate VEGF-A translation not only in EA.hy926 cells but also in primary stromal cells from endometrium from patients with endometriosis and control women. Additional experiments are required in order to elucidate the potential role of miRNA-rich exosomes in this '*in vitro*' model of endometriosis. These promising results really improve the body of endometriosis pathogenesis knowledge that could open up new therapeutic strategies for the treatment of endometriosis through the use of miRNAs.

Supplementary data

Supplementary data are available at http://humrep.oxfordjournals.org/.

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Authors' roles

A.B.-B. developed the idea for the paper, formulated the study design, performed analyses and interpretation of data, and manuscript drafting. S.S.-A. performed analyses and interpretation of data. J.M.-A. performed analyses and interpretation of data and wrote the manuscript. B.A. performed analyses and interpretation of data. R.G.-C. participated in analysis and interpretation of data and participated in a critical revision. M.B.-B. performed analyses and interpretation of data. J.G.-O. provided patients for the study. V.V. participated in a critical revision. A.E. developed the idea for the paper, formulated the study design, participated in analysis and interpretation of data, and wrote the manuscript. J.G.-E. provided patients for the study and participated in a critical revision. C.M. developed the idea for the paper, formulated the study design, performed analyses and interpretation of data, and wrote the manuscript. All authors have approved the final version of the manuscript.

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Conflict of interest

None declared.

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b. Review papers

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Review

miRNAs Regulation and Its Role as Biomarkers in Endometriosis

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Abstract: MicroRNAs (miRNAs) are small non-coding RNAs (18–22 nt) that function as modulators of gene expression. Since their discovery in 1993 in *C. elegans*, our knowledge about their biogenesis, function, and mechanism of action has increased enormously, especially in recent years, with the development of deep-sequencing technologies. New biogenesis pathways and sources of miRNAs are changing our concept about these molecules. The study of the miRNA contribution to pathological states is a field of great interest in research. Different groups have reported the implication of miRNAs in pathologies such as cancer, diabetes, cardiovascular, and gynecological diseases. It is also well-known that miRNAs are present in biofluids (plasma, serum, urine, semen, and menstrual blood) and have been proposed as ideal candidates as disease biomarkers. The goal of this review is to highlight the current knowledge in the field of miRNAs with a special emphasis to their role in endometriosis and the newest investigations addressing the use of miRNAs as biomarkers for this gynecological disease.

Keywords: non-coding RNA; microRNA; biomarker; endometriosis

1. Introduction

Traditionally, in all eukaryotic systems, genes codified proteins, following the central dogma of molecular biology [1] consisting of DNA being transcribed into mRNA and mRNA translated into proteins. The first non-coding RNA (ncRNA) characterized was an alanine tRNA found in baker's yeast in 1965 [2]. Since then, our understanding of the different and capital roles they play in cells and organisms development and functions has enormously increased. ncRNAs are also assumed to be genomic regulators at different levels and, in case of lower level of sequence conservation, are assumed as evolutionary and biodiversity repositories [3].

Nowadays, it is evident that that RNA is not just a simple messenger between DNA and proteins but growing evidence supports new roles for these molecules, such as regulation of genome organization and gene expression. ncRNAs seem to act at many levels playing important roles in epigenetic processes by controlling differentiation and development and they have been related to very different pathologies (Table 1). Short and long ncRNAs are key regulators of gene expression, genome stability, and defense against foreign genetic elements. ncRNAs are encoded in the genome

and never become proteins, demonstrating that the first assumed functions for those molecules have been a lot less than expected [4].

Table 1. Classes of non-coding RNAs (ncRNA).

Symbol	Non-Coding RNAs	Functions
tRNA	Transfer RNA	mRNA translation (structural)
rRNA	Ribosomal RNA	mRNA translation (structural)
miRNA	micro RNAs	Post-transcriptional transposon repression
piRNA	Piwi-interacting RNA	DNA methylation, transposon repression
siRNA	Short interfering RNA	RNA interference
snoRNA	Small nucleolar RNAs	RNA modification, rRNA processing
PROMPT's	Promoter upstream transcripts	Associated with chromatin changes
tiRNAs	Transcripton initation RNAs	Epigenetic regulation
lincRNAs	Long intergenic ncRNA	Epigenetic regulators of transcription
rasiRNA	Repeat associated small interfering RNA	Involved in the RNA interference (RNAi) pathway
eRNA	Enhancer-like ncRNA	Transcriptional gene activation
T-UCRs	Transcribed ultraconserved regions	Regulation of miRNA and mRNA levels
NATs	Natural antisense transcripts	mRNA stability
PALRs	Promoter-associated long RNAs	Chromatin changes
tasiRNA	Trans-acting siRNA	Represses gene expression
lncRNA	Long noncoding RNA	Regulation of gene transcription

2. Non-Coding RNA Identification: The ENCODE Project

ncRNAs are RNA fragments that are transcribed from DNA but are not translated into proteins. The main function of ncRNAs is to regulate gene expression at the transcriptional and post-transcriptional level. ncRNAs can be divided into structural and functional regulatory ncRNAs and, at the same time, functional ncRNAs can be subdivided into two main groups according their length; the short ncRNAs (<30 nts) and the long ncRNAs (>200 nts). The amount of ncRNAs codified in the human genome is unknown; however, recent bioinformatic studies have described the sequence of thousands of them [5]. ncRNAs genes include those that are extremely high expressed and showing essential cell functions such as Table 1 describes. A huge contribution to the identification of untranslated sequences has been the ENcyClopedia of DNA Elements (ENCODE http://www.genome.gov/encode/), released in September 2003. The conclusions from this pilot project were published in June 2007 [6]. ENCODE Project was focused on defining RNA transcripts, transcriptional regulator binding sites, and chromatin states in many cell types by different approaches: (a) genomics, to find functional elements where mutations and knock-down models demonstrate the phenotype associated to the genomic sequence; (b) evolutive conservation, as indicator of functional sequences and (c) biochemical approach in models, to characterize ncRNA activity in specific cell type, condition, and molecular processes [7]. Bioinformatic studies on gene regulation and RNA metabolism have described a new variety of functional non-coding sequences, including promoters, enhancers, silencers, insulators, and ncRNA genes. These non-coding elements are associated with chromatin structures or transcription enhancers displaying, for example, histone modifications, DNA methylation, DNase and transcription factor accessibility [8–13]. Some of them could be considered at some point as 'structural sequences'. YRNAs, for instance, are stem loops essential for DNA replication interacting with chromatin and initiation proteins (including the origin recognition complex) [14,15]. Small RNAs are able to modify chromatin structure and to silence transcription by guiding Argonaute-containing complexes to complementary newly transcribed RNAs scaffolds or to gene promoters [16], mediating histone and DNA methyltransferases recruitment.

3. miRNAs

In 1993, a new possibility was included in the genomic and regulatory scheme. Victor Ambros, Rosalind Lee, and Rhonda Feinbaum, during a study of the lin-4 gene controlling the timing of *Caenorhabditiselegans* larval development, described the first miRNA repressing the lin-14 gene [17]. miRNAs are small (21–22 nts) ncRNAs that regulate gene expression and play fundamental regulatory roles in many biological processes [18–20]. miRNAs can inhibit the translation of hundreds of mRNAs through sequence specific recognition to the "seed sequence", and according to the degree of nucleotide compliment, will raise the inhibition of translation and/or degradation of target molecule of mRNAs [18–21]. Functional analysis of miRNAs have revealed their significant regulatory influence on the expression of target genes involved in both physiological and pathological conditions including gynecological diseases such as endometriosis [22–28].

4. miRNAs Biogenesis

miRNA genes are mainly codified in intergenic or intronic regions of their target genes. In these cases a miRNA gene is transcribed together with its host gene providing a coupled regulation of both. Some pri-miRNA may be codified in the intronic regions of protein and non-protein coding genes or in exons of long non-protein coding RNAs. Consequently, the expression of these miRNAs could be regulated with their host genes [5]. Other miRNA genes show a common promoter forming polycistronic units, containing multiple discrete loops from which mature miRNAs are processed.

In the canonical miRNA biogenesis pathways described in Figure 1, pri-miRNAs are trimmed by the RNAse III Drosha with the help of a double-stranded RNA binding protein: DGCR8. This protein-complex is known as the Microprocessor and yields ~70 nt stem-loop precursors, termed pre-miRNA, with two nucleotides overhanging at the 3′ and a 5′-phospate. The pre-miRNA is then translocated to the cytoplasm by Exportin-5, a Ran-GTP dependent protein [29,30]. From this point on, further steps are common for miRNAs and exogenous siRNAs.

Once in the cytoplasm, the pre-miRNA is trimmed by Dicer, an RNAse III enzyme, in combination with TRBP (transactivation Response RNA-Binding Protein) [31]. As a consequence, the loop sequence of the hairpin is released and Dicer renders a ~22 nt RNA-duplex with short 3′ overhangs. This step defines the 3′ end of the 5′ strand and the 5′ end of the 3′ strand [18]. Then, Dicer transfers the RNA-duplex to an Ago (Argonaute) protein, which forms the nucleus of RISC (RNA-Induced Silencing Complex). At this time, the complex is called the pre-RISC. The mature RISC is achieved once one of the two strands of the duplex is removed; a process termed "strand selection".

The main determinant of this process seems to be a thermodynamic factor, mainly determined by the first four nucleotides of the duplex. Hence, the end with weaker interactions will preferentially unwind and remain as the "guide strand", while the so called "passenger strand" will be discarded [30].

The mature RISC complex is able to scan the cytoplasm searching for mRNA able to pair with the loaded miRNA. The miRNA:mRNA pairing is defined by Watson-Crick interactions between the 3′ UTR (untranslated region) of the mRNA and a short region of nucleotides in positions 2 to 8 of the miRNA known as the "seed sequence". It is worthy to mention that beyond this general principle of miRNA:mRNA interaction, miRNA pairing with the 5′ UTR has also been defined and observed to be of clinical interest [31] and that additional nucleotides outside the seed sequence can also contribute to determine the mRNA fate [18].

It is important to highlight that the DNA sequence is not always the template for the mature miRNA : 6% of human miRNAs suffer RNA editing. In other words, a single pre-miRNA can become multiple mature miRNAs that differ in their length and sequence, named isomiR. The editing process can alter the "seed sequence" conferring different affinity for other targets, modifying the mRNA target selection [5,32]. In this context, the cell-specific expression of different isomiRs implies different protein expression depending on the cell type conferring the biological significance of these miRNA variants. This phenomenon increases the spectrum of miRNA action.

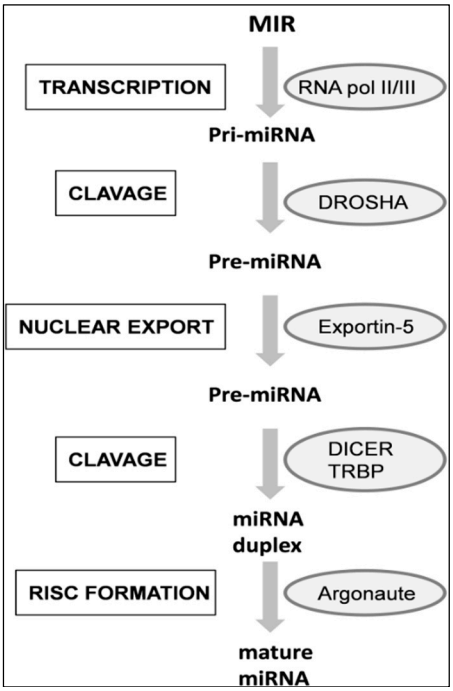

Figure 1. Schematic description of miRNAs biogenesis.

Silencing of mRNAs can be achieved by means of mRNA cleavage or translational repression. Perfect miRNA:mRNA pairing leads to Ago cutting of mRNA approximately at the middle of the miRNA length, whereas imperfect pairing mediates mRNA translational repression by different mechanisms [18].

Apart from the canonical miRNA biogenesis pathway, intronic miRNAs can undergo the "mirtron pathway". In this non-canoncial biogenesis pathway, the spliced intron renders a lariat in which the 3′ brachpoint is ligated to the 5′ end of the intron. Following the action of Ldbr (lariat debranching enzyme) the lariat is converted in a pre-miRNA that can enter the canonical miRNA biogenesis pathway [5,33]. As the vast majority of intronic miRNAs are found on the sense strand it seems plausible that their expression may be related to that of the host mRNA [34,35] in terms of tissue specificity and relative amount [36].

With the avenue of deep-sequencing strategies, the field of miRNAs research has experienced an unprecedented growth in terms of genetic origins, biosynthetic pathways, and sequence variants [5]. As a result, several ncRNAs have been identified as sources of miRNAs, including snoRNA, lncRNA, and tRNA genes with Drosha- and/or Dicer-independent biogenesis [5].

5. miRNAs Nomenclature

The recent advances in high-throughput sequences applied to the miRNA discovery have enormously challenged criteria for miRNA annotation. Nomenclature rules are currently defined by miRBase 21 [37] and the mature form of the miRNA fit the form hsa-miR-XX-3p/5p, where the prefix refers to the species (e.g., hsa- for Homo sapiens). When it is written in capitalized letters, "MIR", refers to the gene that encodes them; and pre-miRNA and pri-miRNA are named as "mir-". Distinct precursor sequences and genomic loci expressing identical mature sequences get names of the form hsa-mir-121-1 and hsa-mir-121-2 and adding letters as suffixes denotes mature sequences closely related (hsa-miR-121a and hsa-miR-121b) named miR families. Cloning studies sometimes identify two mature sequences originated from the same pre-miRNA. The ratio between the two opposite mature strands can vary depending on developmental stage, being differentially expressed in distinct tissues or cell types, as well as in pathological conditions [5,30]. Previous nomenclature

versions identified the less expressed strand as asterisk * (hsa-miR-XX *). However, recent studies have demonstrated that both strands are functional and the ratio between strands depends on the cellular type or status, the annotation criteria was appropriately changed to the current 5p-/3p-end. Apart from the aforementioned miRNA nomenclature, miRBase also identifies mature miRNAs with a MIMAT accession number. From our own experience, we do recommend authors to refer to studied miRNAs in their manuscripts with the current -3p or -5p suffix and also to include the miRBase MIMAT reference and oligonucleotide sequence in order to avoid future misunderstanding that further nomenclature modifications could introduce.

In the light of current discoveries in miRNA origins, biosynthetic pathways, and sequence variants, Desvignes and co-workers proposed a revised miRNA nomenclature criterion in the aim of encompassing recent findings in the field. The authors proposed to modify the miRNA nomenclature not only based on biogenesis but also on their function [5].

6. Studying miRNAs

In a classic study of miRNAs, the first aim is to assess the miRNA expression profile comparing a pathological group to a control one. The second step in order to corroborate the profiling results is to validate the expression of some of selected miRNAs in a larger cohort of samples. Finally, it would be interesting to prepare functional assays in order to validate the regulation of a specific mRNA translation by the selected miRNA. Summary of technologies applied in miRNA discovering are listed in Figure 2.

The most employed techniques in order to assess miRNA expression profiles are next generation sequencing and microarrays.

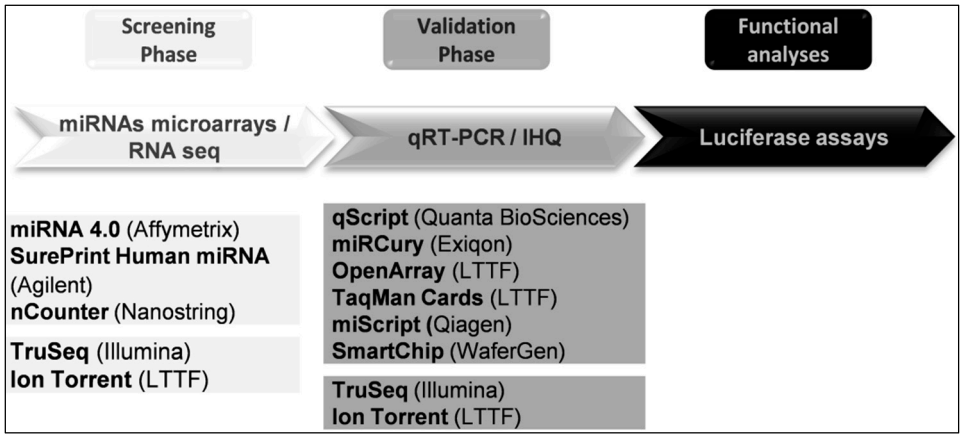

Figure 2. Workflow in a classical study on miRNAs.

6.1. RNA Sequencing

Next Generation Sequencing (NGS) was initially developed for sequencing genes faster, cheaper and deeper. The ability to read millions of short fragments of DNA was soon moved into a new methodology, RNAseq, which includes RNA sequencing and quantification. Total RNA from samples is digested and retrotrasncribed through ligation of universal primers. cDNA products are then ligated to primer adaptors for NGS and amplified and isolated in single sequencing reactions on a surface containing millions of "nanopore" sequencing reactors. Second generation sequencers are able to detect millions of isolated sequencing reactions and to generate data related to nucleotide sequences and the number of reads of each sequence. The bioinformatic software associated with the NGS sequencer is able to align each short RNA fragment with their corresponding genomic region, building a complete transcript read. Analogous to qPCR, the number of reads obtained in RNAseq protocols is correlated to the original amount of cell RNA together with sequence variant detected

in the alignment. As limitations, the sensibility of NGS is linked to coverage and throughput, so the presence of over-expressed miRNAs can reduce the ability for detecting miRNAs very low expressed.

6.2. miRNA Microarrays

Microarrays have been a revolution in genomic and proteomics fields because of their ability to simultaneously detect thousands of molecules. Thereafter, expression assessment moved from particular genes to whole genomic profiles in which miRNA profiles helped to decode the real complexity of pathological models [38]. Microarray technology is based on the link of millions of genomic fragments on a glass support that are used for sample hybridization (Affymetrix, Agilent, Illumina, NanoString). Samples are fluorescently labeled prior to hybridization. Thus, relative signal on each probe reflects the original amount of miRNA in the studied sample. Hundreds of new ncRNAs are described on an annual basis. For this reason, updating and re-designing of non-coding RNA microarray platforms is mandatory. miRNA expression profiles obtained from microarray platforms should be validated by qPCR in a larger number of samples.

Both techniques have demonstrated their ability in measuring a large number of miRNAs, generating a wide amount of results in the discovery phase. However, nowadays the economic cost is still too expensive to employ them in the validation step. Therefore, quantitative PCR is confirmed to be the most accurate technique to validate results from RNAseq or microarrays

6.3. Quantitative PCR or Real-Time PCR

Quantitative PCR (qPCR) or Real-Time PCR (RT-PCR) is a fast, easy, and affordable technique for quantification of miRNAs. Different names depend on applications or thermocycler platforms employed. PCR allows working with very few amount of starting RNA. The first step consists in the synthesis of complementary DNA to miRNA through an adapter following a retrotranscription protocol (RT-PCR). The exponential amplification process in RT-PCR is an extremely sensitive and accurate method for detecting molecules at very low level, thus becoming the gold standard method for quantification of miRNAs in biofluids such as plasma or serum. Few limitations can be attributed to this technique, for example, the limited number of molecules detected depending on plate design (96- or 384-well plate). Pre-amplification and amplification increase sensitivity quantification can be biased in low expressed sequences due to diverse protocol steps. On the other hand, normalization can be difficult because, commonly, "housekeeping" molecules that are used as normalizers can be not stably expressed in some models or pathologies. In any case, so far, qPCR is the reference method for expression validation of other techniques in miRNA research [39]. Nowadays, some high-throughput PCR designed plates are called arrays (Applied, Qiagen, Exiqon). These plates include a customized selection of probes ready-to-use in order to quantify a high number of miRNAs from a single sample reducing technical variations.

6.4. In Situ Hybridization (ISH) and Live Cell miRNA Detection

Some companies already specialized in miRNA detection have also developed fluorescent or antibody-conjugated colored-coupled probes for *in situ* miRNA detection (Exiqon, Merk-Millipore). These methodologies allow localizing miRNA molecules in cells or tissues, helping to better characterization of its biogenesis, pathways, and activity. Fluorescent-labeled probes show miRNA localization in fixed or live cells and allow, in some assays, to perform flow sorting of cells expressing concrete regulators [40]. During ISH, a locked nucleic acid (LNA) probe hybridizes to the target sequence at elevated temperature, and then the excess probe is washed away. LNA probes are highly sensitive and have been assayed for therapeutically miRNA antisense therapies [41].

7. miRNAs as Biomarkers

"Biomarker" definition has been revised by the Biomarker Definitions Working Group in 2001 [42] as "a characteristic that is objectively measured and evaluated as an indication of normal biologic

processes, pathogenic processes, or pharmacologic responses to a therapeutic intervention". In the "omics" era biomarkers can include clinical scoring systems, proteins, gene expression measurements of mRNA, miRNA, DNA, genetic variants of DNA, or metabolites [43].

With the aim to better understand the pathophysiology of different diseases, several authors have reported miRNA expression profiles characteristic of some pathologies (cardiovascular diseases, cancer, diabetes, sepsis, gynecological diseases) [25,26,28,44,45]. The first approach to define a pathological miRNA expression profile is to assess the miRNA expression pattern in the pathological tissue in order to clarify the molecular mechanisms underlying the disease. In this context, miRNAs have reportedly been found in different body fluids, from urine, serum, and plasma to cerebrospinal fluid [46,47].

The presence of miRNAs in different biofluids could be explained by different mechanisms [39,43,48,49]: (a) passive release of miRNAs as consequence of tissue injury, chronic inflammation, cell apoptosis or necrosis, or from cells with a short half-life, such as platelets; (b) active secretion via cell-derived microvesicles including exosomes microparticles and apoptotic bodies [50–52]; (c) active secretion by cells in miRNA-protein complexes: High Density Lipoprotein (HDL) and Low Density Lipoprotein (LDL) [53] and Ago2 [54]. Interestingly, Arroyo and co-workers suggested that Ago2-associated miRNAs could be ready to regulate gene expression in recipient cells [54]. Mechanisms (b) and (c) could also offer a rationale for the elevated stability of miRNAs in an RNAse-rich circulation [49]. In light of this evidence, some authors proposed an hormone-like mechanism of action for these miRNAs [48,49] and a role into cell-to-cell communication [55–58].

Circulating miRNAs, such as the miRNAs released by cancer cells, can bind to Toll-like receptors (TLRs) of immune cells, such as TLR7 in mice or TLR8 in human, to activate NFκB [59,60]. This mechanism could partially explain inflammation related to cancer.

Importantly, Mitchell and co-workers showed the potential of blood miRNAs as biomarkers for prostate cancer [61], paving the way for further characterizations in other pathologies. In addition, the authors demonstrated that miRNAs were protected from endogenous RNAse activity [61].

8. miRNAs Role in Endometriosis

Different groups, including ours, have studied the potential role of miRNAs in the endometriosis development over years. miRNAs raise as potent regulators of gene expression in the most important systems involved in the pathogenesis of endometriosis. As Figure 3 details, cell survival, matrix remodeling, proliferation, and angiogenesis are essential systems in the pathophysiology of this disease and all of them are potentially regulated by miRNAs [21,22,25,28,62].

9. Endometriosis

Endometriosis is a benign estrogen-dependent inflammatory disorder characterized by the presence of endometrial-like tissue outside the uterus. Endometriosis-lesions can be found on the peritoneum (peritoneal lesions), on the ovary either as superficial implants or as endometriotic cysts, and as deeply infiltrative lesions that might extend to the bowel, bladder, and ureter. Pelvic adhesions are often associated with the aforementioned lesions [63]. These lesions are responsible for the main symptoms of endometriosis, pelvic pain and infertility [64]. Whereas innervation at the site of endometriotic lesions is involved in pelvic pain [65], inflammation has been also associated with infertility, what could be explained by a diminished oocyte quality because if their development in an unfavorable environment [66] and also a compromised endometrial receptivity [67].

Endometriosis has been classified as a tumor-like condition by the World Health Organization Histologic Classification of Ovarian Tumors [68]. Indeed, endometriosis shares common features with cancer, as increased local estrogen production, reduced apoptosis, pro-survival, inflammation, tissue invasion, induction of angiogenesis, and dysfunction of immune cells [69]. Since Sampson reported the first case of suspected malignant transformation of ovarian endometriosis [70], several studies have focused on the relationship between endometriosis and gynecological cancers, especially endometrioid and clear cell ovarian carcinoma [71–74]. However, in a recent review, Guo pointed that existing data

is not enough to establish a doubtless causality and highlighted the need for further molecular studies in order to establish an unequivocal phylogenetic relationship between both conditions [69].

Despite its high prevalence and incapacitating symptoms, the exact etiopathogenic mechanism of endometriosis remains unsolved. Burney and Giudice reviewed the theories purposed in recent years with the aim of providing a plausible etiopathogenic mechanism for endometriosis [75]. However, nowadays, the most accepted theory is by far Sampson's retrograde menstruation proposal, which points that during menstruation, endometrial fragments could migrate through fallopian tubes and reach the peritoneum, being capable to attach, survive, and implant at different locations [70]. It has been demonstrated that all these mechanisms responsible for endometriosis development can be regulated by miRNAs as Figure 3 shows.

10. Studying New Biomarkers of Endometriosis

The current gold standard for the diagnosis of endometriosis is laparoscopic examination with histological confirmation of glands and/or stroma in the excised lesions [76]. The need for surgical procedure for diagnosis together with the fear of a cancer diagnosis and the assumption of dysmenorrhea as a normal event could explain the aforementioned delay in time to diagnosis [77]. Taking into account that endometriosis has been reported to be progressive in up to 50% of women [78] and more advanced in women with delayed diagnosis [79] efforts are conducted to achieve a noninvasive diagnosis. In this context, several approaches have been undertaken, such as symptom-based tests [80], or blood tests [81–83], but so far neither a non-invasive nor a minimally invasive test has been achieved, remaining as a priority in endometriosis research [84]. Therefore, an ideal test for diagnosis of endometriosis should diagnose patients at initial stages with high sensitivity and specificity.

Due to the anatomical location of this condition, several closely related biofluids have been proposed as a source for noninvasive biomarkers of endometriosis, for instance: urine, plasma/serum, and menstrual blood. In addition, the finding that retrograde menstruation is present in 90% of women but not all of them suffer from endometriosis [67,85] suggests that molecular differences between eutopic endometrium from women with and without endometriosis may exist that lead to the development of the condition in certain women but not in others [67]. As a consequence, if these molecular differences were found to be pathognomonic of the condition they could also provide an opportunity to be considered as biomarkers in biopsied tissues obtained via a minimally invasive procedure.

In the field of miRNAs, differences in miRNA expression between endometriotic lesions and eutopic endometrium from women with endometriosis have been reported [27,86] but few studies have focused in differences between eutopic endometrium from women with and without endometriosis [21,27,87].

Burney *et al.* published one of the first studies addressing the miRNA expression profile in the endometrium of women with and without endometriosis [21]. In this study, miRNA arrays were performed and after qRT-PCR validation, the authors reported a downregulation of four miRNAs (miR-34c-5p, miR-9, miR-9 *, miR-34b *) in the eutopic endometrium from women with endometriosis compared to control endometrium. According to the miRNA regulatory mechanisms, downregulated levels of a miRNA entails the upregulation of its target mRNA translation. Laudanski *et al.* conducted a study enrolling 25 endometriosis-free women and 21 patients with ovarian endometriosis in which the expression of 667 human miRNAs was examined by means of PCR arrays. Validation of the results led to the corroboration that miR-483-5p, a regulator of IGF2, and miR-629-3p, involved in inflammation, were downregulated in the eutopic endometrium of patients in comparison to controls. The authors pointed to the idea that dysregulation of these genes could contribute to the overgrowth of endometrial tissue outside the uterus [87].

Human endometrium is a unique tissue that undergoes complex molecular, cellular, and functional changes on a cyclic basis under ovarian hormone regulation [88–90]. These changes are

essential for uterine receptivity and can be grouped in three distinct phases: proliferative, secretory, and menstrual [90]. Some authors have described that miRNA expression vary across the menstrual cycle [24]. Particularly, miRNAs targeting several cell cycle regulators were over-expressed in the secretory phase [23].

Angiogenesis also plays an important role in the pathogenesis of endometriosis, due that ectopic lesions require neovascularization to proliferate, invade the extracellular matrix and proliferate [27,28,91]. Both Vascular Endothelial Growth Factor A (VEGF-A) and Thrombospondin-1 (TSP-1) represent the most potent pro- and anti-angiogenic factors, respectively, and have been involved in the pathology of endometriosis [92]. Our research group has reportedly observed an increase in the expression of angiogenic and proteolytic factors in endometrial tissues from patients with endometriosis [93,94] and we have suggested that this increase might contribute to the invasive potential of endometrial cells.

The miRNA regulation of angiogenesis has been long reported in several pathologies, including endometriosis [27,28,86,87,95,96]. Two different groups have reported that the angiogenesis regulators, miR-17-5p and miR-20a, are downregulated in the ovarian endometrioma compared to eutopic endometrium [24,87]. The miR-17-92 cluster, also known as oncomir-1, encodes six mature miRNAs (miR-17, miR-18a, miR-19a, miR-19b, miR-20a, and miR-92a) [97]. Recently, our group has reported that the miR-17-92 cluster increases tumour neovascularization by decreasing TSP-1 expression [98]. Therefore, and due to the miRNA mechanism action, a decrease in miR-17-5p levels involve post-transcriptional upregulation of TSP-1 levels. This mechanism may reduce the angiogenic activity in the ovarian endometrioma; therefore, it could explain the low ability in the extracellular matrix invasion of this tissue observed in these ectopic lesions where frequently the ovarian tissue remains preserved.

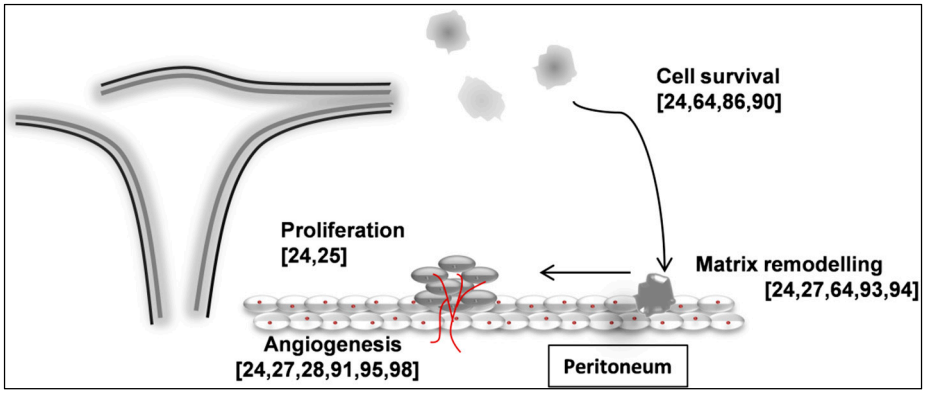

Figure 3. Main systems affected in endometriosis and the references for the most relevant studies about the role of miRNAs regulating them in this disease in square brackets. Red lines represent new blood vessels in formation and red dots cells nuclei.

11. Circulating miRNAs as Biomarkers of Endometriosis

The study of circulating miRNAs as biomarkers of endometriosis is an emerging field of research, and to date only few studies have been published both in serum [82] and plasma [81,83].

Wang *et al.* [99] first performed a circulating miRNA array profiling in two pools of sera from 10 patients with endometriosis and 10 control women. After validation of results from array by qRT-PCR in sera from 60 patients and 25 control women, the authors found that miR-199a and miR-122 levels were upregulated and miR-145 *, miR-141 *, miR-542-3p, and miR-9 * downregulated in samples from patients in comparison to control women and could therefore serve as biomarkers of the disease.

Shortly after, another study in plasma was conducted by Jia *et al.* [81]. 23 women with histologically proven endometriosis and 23 endometriosis-free controls were enrolled in the study and a miRNA microarray profiling was performed. Three out of the six miRNAs selected for validation by

qRT-PCR (miR-17-5p, miR-20a, and miR-22) were proven to be significantly downregulated in patients and useful to discriminate women with endometriosis from patients.

In 2015, two studies have been published, extending the evidence of miRNAs as putative biomarkers of endometriosis in non-invasive biofluids. In the first case, levels of previously endometriosis-associated miRNAs, miR-135a,b and let-7a-f, were quantified in sera of 24 endometriosis patients and 24 control women. By means of a logistic regression approach, researchers found that combining levels of let-7b, let-7d, and let-7f in the proliferative phase obtained the highest area under the curve value in order to discriminate patients with endometriosis from control women. [82]. Of note, several miRNAs were found to be differently expressed depending on the phase of the menstrual cycle in patients but not in controls, in agreement with previous reports [100]. Finally, Rekker *et al* [83] performed the last published study regarding circulating miRNAs as biomarkers of endometriosis. Based on previous literature, authors selected three miRNAs from the miR-200 family (miR-200a-3p, miR-200b-3p, and miR-141-3p) whose expression was assessed in plasma samples from 61 patients and 65 control women. The expression of all three miRNAs was downregulated in patients and miR-200a-3p and miR-141-3p showed the highest potential as noninvasive biomarkers for this benign condition. Remarkably, authors also analyzed variations of the levels of the three miRNAs of interest with time of sampling (morning/evening) finding lower levels in evening samples, perhaps due to circadian fluctuations in their expression. This is an interesting approach and points to the time of sampling as an important factor to be taken into account when performing circulating miRNAs studies.

12. Conclusions

Among the epigenetic players, miRNAs have emerged as pivotal post-transcriptional regulators. To do this, these small non-coding RNAs bind to their target mRNAs and inhibit the translation process. The involvement of miRNAs in different pathological conditions has been well established and the miRNA expression profiles have been performed in biopsies from different diseases, including gynecological pathologies as endometriosis. Despite being a benign gynecological pathology, endometriosis deeply impairs the quality of life of affected women in terms of pain and infertility. Nowadays, the gold standard to diagnose endometriosis is laparoscopy. For this reason, several groups including ours are focused on characterizing a non-invasive or semi-invasive biomarker for the diagnosis of endometriosis at initial stages that overcomes the need for the current laparoscopy. Recently, circulating miRNAs have emerged as attractive molecules to be considered as biomarkers [45], although deeper studies are required in order to characterize and validate a miRNA-based diagnostic tool. It is important to highlight the important differences in experimental design and preanalytical protocols among different studies evaluating the same pathology; making it difficult to compare results [40]. For all these reasons, the World Endometriosis Research Foundation (WERF) has published recommendations in order to standardize the data and sample collection, processing and storage [101–104] and reduce the heterogeneity and improve the reproducibility between studies as summarize the Figure 4. It is essential to unify every step in endometriosis research. The first one is data collection; for this purpose the WERF have elaborated a guide for surgical data collection as well as video/photo of symptom documentation [101,102]. Regarding the study of biofluids, the WERF has defined the protocol in order to standardize the biospecimen collection, processing, and storage [103]. Finally, the collection and storage of tissue samples have also been standardized according to the consensus document [104]. All these documents [101–104] allow for unifying the studies performed around the world about endometriosis. The aforementioned guides could be also useful for the study and validation in other diseases; these steps avoid the publication of dissimilar studies performed in the "same" disease but employing different protocols. This could be a simple way to obtain robust conclusions and be able to standardize new biomarkers. However, the study of miRNAs as biomarkers implies additional considerations. As it has been previously described, miRNAs are very stable circulating molecules; however heterogeneity among patients seems to be substantial. This feature is even more evident when results from one study are replicated by other group. Based on our own

experience, pharmacological treatments, clinical conditions, or even diet can affect severely miRNA expression profiles in plasma.

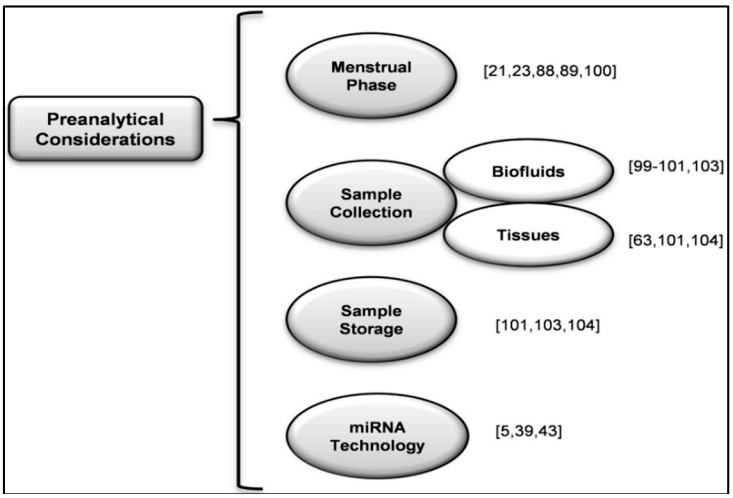

Figure 4. Preanalytical considerations for the miRNA study and relevant literature in square brackets in order to obtain robust conclusions in the standardization of new biomarkers in endometriosis.

In conclusion, miRNAs have emerged as new biomarkers valid for diagnostics or prognostics of several diseases. However, standardization in sample and clinical data collection; sample processing and storage; and technical protocols become essential for saving time and money in the assessment of miRNAs as biomarkers.

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REVIEW ARTICLE

Translating cancer epigenomics into the clinic: focus on lung cancer

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Epigenetic deregulation is increasingly being recognized as a hallmark of cancer. Recent studies have identified many new epigenetic biomarkers, some of which are being introduced into clinical practice for diagnosis, molecular classification, prognosis or prediction of response to therapies. O-6-methylguanine-DNA methyltransferase gene is the most clinically advanced epigenetic biomarker as it predicts the response to temozolomide and carmustine in gliomas. Therefore, epigenomics may represent a novel and promising tool for precision medicine, and in particular, the detection of epigenomic biomarkers in liquid biopsies will be of great interest for monitoring diseases in patients. Of particular relevance is the identification of epigenetic biomarkers in lung cancer, one of the most prevalent and deadly types of cancer. DNA methylation of SHOX2 and RASSF1A could be used as diagnostic markers to differentiate between normal and tumor samples. MicroRNA and long noncoding RNA signatures associated with lung cancer development or tobacco smoke have also been identified. In addition to the field of biomarkers, therapeutic approaches using DNA methylation and histone deacetylation inhibitors are being tested in clinical trials for several cancer types. Moreover, new DNA editing techniques based on zinc finger and CRISPR/Cas9 technologies allow specific modification of aberrant methylation found in oncogenes or tumor suppressor genes. We envision that epigenomics will translate into the clinical field and will have an impact on lung cancer diagnosis/prognosis and treatment. (Translational Research 2017;■:1–17)

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Abbreviations: BAL = bronchoalveolar lavage; ChIA-PET = chromatin interaction analysis with paired-end tag; ChIP-seq = chromatin inmunoprecipitation and massively parallel sequencing; circ-ncRNA = circulating noncoding RNA; CRC = colorectal cancer; CRISPR-Cas9 = clustered, regularly-interspaced short palindromic repeats-associated protein 9; CSC = cigarette smoke condensate; ddPCR = droplet digital PCR; DNMT = DNA methyltransferase; DNMTI = DNA methyltransferase inhibitor; EMT = epithelial-to-mesenchymal transition; HDAC = histone deacetylase; HDACI = histone deacetylase inhibitor; HMA = hypomethylation agents; HMT = histone methyltransferase; lncRNA = long noncoding RNA; miRNA = microRNA; MGMT = O-6-methylguanine-DNA methyltransferase; ncRNA = non-coding RNA; NGS = next generation sequencing; NSCLC = non-smallcell lung cancer; PAM = protospacer adjacent motif; PCa = prostate cancer; PTM = post-translational modifications; SCC = squamous cell carcinomas; SCLC = small-cell lung cancer; sncRNA = short noncoding RNA; THU = tetrahydrouridine; TSG = tumor supresor gene; VEGF-A = vascular endothelial growth factor A; ZFP = zinc finger proteins

INTRODUCTION

It is currently acknowledged that epigenetic alterations play a crucial role in cancer development. Initially proposed by Riggs et al in 1996, the term epigenetics describes "the study of mitotically and/or meiotically heritable changes in gene function that cannot be explained by changes in DNA sequence." A decade later, Bird proposed a refined definition of epigenetics as "the structural adaptation of chromosomal regions so as to register, signal or perpetuate altered activity states."[1] Epigenetic mechanisms include DNA methylation/demethylation, histone modifications, chromatin remodeling, and expression of noncoding RNAs (ncRNAs). Although these changes have been traditionally identified in one-at-a-time experiments, the implementation of novel genome-wide technologies are now producing massive amounts of data that will improve our understanding of tumor development and progression. Moreover, analysis of epigenetic changes in single genes and gene signatures either in tumor specimens or liquid biopsy can be useful in clinical settings for the molecular subclassification of tumors, prognosis, prediction of response to therapy, and follow-up of the patients after therapy.[2] Of particular interest is the detection of epigenomic biomarkers in liquid biopsy using novel ultrasensitive techniques, since this would allow the minimally invasive monitoring of the patients. In addition, recent studies have shown that epigenetic alterations can be targeted not only in hematological malignancies, but also in solid tumors. This review focuses on lung cancer, a type of malignancy that causes the highest number of cancer-related deaths worldwide.[3] Since lung tumors in advanced stages are rarely cured, early detection is critical to improving survival. In the last decade, several clinical trials of annual screening with low-dose computerized tomography have shown a reduction in cancer mortality.[4,5] However, several potential harms have been shown such as the risk of irradiation and overdiagnosis which could affect patients' quality of life, increase patient anxiety, and costs.[6] There is an urgent need to identify and validate biomarkers able to select individuals with a high risk for developing lung cancer. Furthermore, in recent years, targeted therapies and immunotherapy have improved the prognosis of patients with advanced non-small cell lung cancer (NSCLC), even though not all patients respond and those ultimately progress because of the development of resistances. Biomarkers to predict those patients with a higher probability of responding to therapy and those who will develop early resistance are clearly needed. Epigenetic biomarkers, either in combination with genetic biomarkers or alone, may offer a great opportunity for better monitoring and managing of patients.

EPIGENETIC PLAYERS: DNA METHYLATION, HISTONE MODIFICATIONS, AND NONCODING RNAS

DNA methylation. Among epigenetic marks, DNA methylation is by far the most widely studied. It is a covalent modification at the 5' carbon of cytosines catalyzed by a family of enzymes: the DNA methyltransferases (DNMTs). Whereas DNMT1 recognizes hemimethylated DNA and is classified as maintenance methyltransferase, DNMT3a and DNMT3b can introduce methylation marks without a template and are therefore termed de novo methyltransferases. Deviations in DNA methylation patterns can occur via gain (hypermethylation) or loss (hypomethylation) of methylation marks with respect to methylomes defined as normal.[7] In a cancer context, hypermethylation at CpG islands of gene promoters is associated with the repression of tumor suppressor gene (TSGs) expression.[8,9] Conversely, genome-wide hypomethylation in cancer cells has been linked to expression of proto-oncogenes, genomic instability,[10] and malignant transformation of tumors; a feature that increases with cancer progression.[11]

A common undertaken approach in epigenetics research has been the quantification of the enzymatic levels of the machinery responsible for DNA methylation, with the rationale that altered levels in these proteins would lead to consequent changes in global methylation patterns. Focusing on pulmonary pathology, increased DNMT1 protein levels after tobacco carcinogen exposure have been correlated with promoter hypermethylation of the cadherins *Dal1*, *E-cadherin*, *H-cadherin*, and *protocadherin-10* in human bronchial cell lines. These findings have established overexpression of *DNMT1* and repression of TSG as early events in lung carcinogenesis. Interestingly, levels of the other DNMTs (*DNMT3a* and *DNMT3b*) remained invariable.[12] Furthermore, *DNMT1* and *DNMT3b* overexpression in NSCLC has been associated with hypermethylation and downexpression of the TSG *p16*.[13] Recently, Husni et al. postulated *DNMT3a* overexpression to be an independent prognostic marker in lung adenocarcinoma and an indicator of favorable prognosis, given its association with the histologic noninvasive type and lepidic subtype.[14]

Before the advent of the omics era, methylation levels in specific set of genes had been explored both for their role as a mechanism of disease but also for their potential as biomarkers for diagnosis, prognosis, and response to chemotherapy. Hypomethylation of the repetitive element *LINE-1* has been postulated to be an independent marker of poor prognosis in stage IA NSCLC. In squamous cell carcinomas (SCCs), a subtype of NSCLC, hypomethylation of the intrinsic *P2* promoter of *p73* and the subsequent increased expression of the antiapoptotic *ΔNp73* protein isoform was found to be a frequent event in this type of malignancy.[15] Given that hypomethylation of *P2* and *LINE-1* are highly correlated, overexpression of *ΔNp73* could be a consequence of overall DNA hypomethylation.[16]

Methylation status is significantly different in NSCLC samples than in normal lung for several genes, including *P16*, *RASSF1A*, *RAR*, *APC*, *CDH1*, *MGMT, SHOX2,* and *TMPRSS4*. As for diagnosis, Zhao et al conducted a meta-analysis in which they corroborated the previously reported diagnostic value of *SHOX2* methylation in lymph node and lung cancer tissue biopsies and in noninvasive samples.[17] Moreover, regarding the potential prognostic value of methylation marks in biopsies, our group has recently validated both the hypomethylation at the *TMPRSS4* gene promoter and the overexpression of this prometastatic serine protease as independent predictors of poor prognosis in patients with early-stage SCC.[18] In addition, DNA methylation status at specific promoters could also serve as a predictor of responsiveness to chemotherapy. A well-known example is provided by the DNA repair gene *MGMT* in glioblastoma patients treated with alkylating agents. *MGMT* acts by removing promutagenic alkyl groups from the O6 position of guanine in DNA, thus reducing the cytotoxic activity of alkylating chemotherapeutic agents in tumors.[19] Patients not expressing *MGMT* due to hypermethylation at its promoter survived longer and responded better to temozolomide and carmustine.[20] These findings support the role of *MGMT* methylation status as a predictor of the responsiveness of the tumor to alkylating agents.[21]

Histone modifications. In addition to DNA methylation, covalent post-translational modifications (PTMs) at the protruding N-terminal tails of histones can alter internucleosomal interactions and affect chromatin structure and thus gene expression. Nucleosomes, the basic units of DNA packaging, are composed of 147pb of DNA wrapped around an octamer of the so-called core histones (dimers of H2A, H2B, H3, and H4), with H1 acting as a linker histone. Several chemical groups can be reversibly added to the basic N-terminal tails of these proteins, either affecting the electrical charge (as is the case with acetylation, phosphorylation, and deimination) or without affecting the electrical charge (as in methylation, ADP-ribosylation, ubiquination, biotinylation, and SUMOylation) of histones.[22,23] The myriad of possibilities of combination of distinct chemical modifications in histone tails associated with open/closed states of chromatin is the basis of the hypothesis of the histone code, proposed by Strahl and Allis almost 2 decades ago.[24] Given that histone modifications have context-dependent effects, with a complexity similar to a "language," several authors are considering the concept of epigenetic language, rather than that of epigenetic code.[25,26]

Due to its potential in clinical applications, we will focus in this review on the histone PTMs acetylation/deacetylation and methylation/demethylation, carried out by the "writer" enzymes histone acetyltransferases and histone methyltransferases (HMTs), and the "eraser" enzymes histone deacetylases (HDACs) and histone demethylases, respectively. As was the case for DNMTs, the levels of "writer" and "eraser" enzymes for histone PTMs have also been extensively quantified in a wide number of studies. For instance, overexpression of HDAC has been documented in several malignancies, including endometrial,[27] pancreatic,[28] and gastric[29] carcinomas. More concretely, Jiao et al. showed that the levels of HDAC3 are elevated in pancreatic cancer tissues and cell lines.[28] Importantly, HDAC3 functions as an oncogenic protein and has been found to promote proliferation, migration and

invasion, and the repression of *P53*, *P27,* and *BAX* tumor suppressor genes that this protein mediates.

In several types of cancer, genome-wide analyses have revealed specific modifications associated with tumors.[9] A study conducted in 25 human cancer cell lines (including leukemia, lung and cervix, among others) and 36 human primary malignancies (including but not exclusively lymphoma and colorectal cancer [CRC]) highlighted the loss of monoacetylation at H4K16 and trimethylation at H4K20 as a hallmark of cancer. Interestingly, these marks occur at repetitive regions where hypomethylation is present,[30] exemplifying the interplay between distinct epigenetic players. In lung cancer, increased levels of acetylation marks in H4K5/H4K8 and loss of trimethylation of H4K20 have been documented in NSCLC.[31] Interestingly, H4K20 loss of trimethylation characterized a subpopulation of early-stage (I) lung adenocarcinomas associated with poor prognosis.[32]

Although a vast amount of knowledge has been gained from research in histone PTMs, the functions they mediate is beginning to be unraveled. Further investigations will not only clear the path for a more precise knowledge of the cooperative action of coexisting histone PTMs, but also of the dialog between histone PTMs and other epigenetic players, such as DNA methylation and noncoding RNAs.

Noncoding RNAs. A great percentage (98%) of our genes renders transcripts that will never become proteins, but rather have a regulatory function in both physiological and pathophysiological states. These transcripts are termed noncoding RNAs (ncRNA) and can be classified, according to their length, into short ncRNAs (sncRNAs, <200 nt) and long ncRNAs (lncRNAs, >200 nt).[33,34]

MicroRNAs (miRNAs), a class of sncRNAs (19–22 nt), were first discovered in *Caenorhabditis elegans* 2 decades ago[35] and have attracted great interest in research since that. miRNAs regulate gene expression by specific mRNA pairing and prevention of its translation. Different miRNA profiles have been linked to the etiology, progression, and prognosis of several malignancies,[36,37] leading to the classification of specific miRNAs either as "oncomiRs" or "tumor suppressors," depending on their targets.[38] For instance, the polycistronic *miRNA 17–92* cluster, also known as *oncomiR-1*,[39] has been reported to be overexpressed in SCLC, acting as the enhancer of proliferation and the inhibitor of apoptosis.[40] Interestingly, members of the *miRNA 17–92* cluster are also capable of increasing angiogenesis in cancer and other conditions.[38,41] Moreover, reduced expression of the miRNA family *let-7* in SCLC, induced by *Lin-28*, has been observed to enhance cell proliferation through the upregulation of *CDC25A*.[33] On the other hand, lncRNAs have been observed to play a role in cancer metastasis interacting with epigenetic effectors and controlling splicing. For instance, overexpression of the metastasis-associated lung adenocarcinoma transcript 1 (*MALAT1*) has been associated with poor survival parameters in different neoplasms such as NSCLC, bladder, and laryngeal squamous carcinoma.[42]

The discovery of ncRNAs in biofluids[43,44] as cell-free circulating ncRNAs (circ-ncRNAs) has paved the way for their use as potential noninvasive biomarkers in a wide variety of malignancies, as will be discussed below. Importantly, circ-ncRNAs in cancer may not only be informative of the presence of the tumor but rather reflect its biology, serving not only as diagnostic tools but also as valuable markers of response to treatment and prognosis.[44-46]

EPIGENOMIC PROFILING IN CANCER

The application in the epigenetic field of genome-scale analysis techniques, such as next generation sequencing (NGS) and microarray systems, has opened up the new era of epigenomics (Fig 1). The DNA methylation microarray Infinium HumanMethylation450 BeadChip, that permits the analysis of over 480,000 CpGs (450K), has proved to be useful in cancer for addressing genome-wide DNA methylation profiling not only in cell lines but also in large cohorts of sample patients.[47,48] This methodology offers high reproducibility, speed, and a reasonably low price per sample.[49] Recently, a new version of this platform has been developed: the Infinium DNA MethylationEPIC BeadChip microarray. This new system covers over 850,000 CpG methylation sites (850K) with >90% of the 450K sites plus an additional 333,265 CpGs located in enhancer regions identified by the ENCODE and FANTOM5 projects. Importantly, the 850K array presents high reproducibility in the analysis of fresh frozen and formalin-fixed paraffin-embedded samples.[50]

It is also currently possible to perform a rapid unbiased analysis of the total DNA methylome at a single-base resolution by means of whole genome sequencing.[51,52] This methodological approach has been shown to be useful in the genome-wide analysis of the epigenome in different tumor samples, including the analysis of single cells and biological fluids such as plasma.[53-55] Although the use of NGS in methylome studies has increased in the last 2 years, most translational cancer studies are still being performed using microarray platforms, primarily due to the excellent cost/quality ratio.[56]

Regarding histone modifications, the combination of chromatin immunoprecipitation and massively parallel

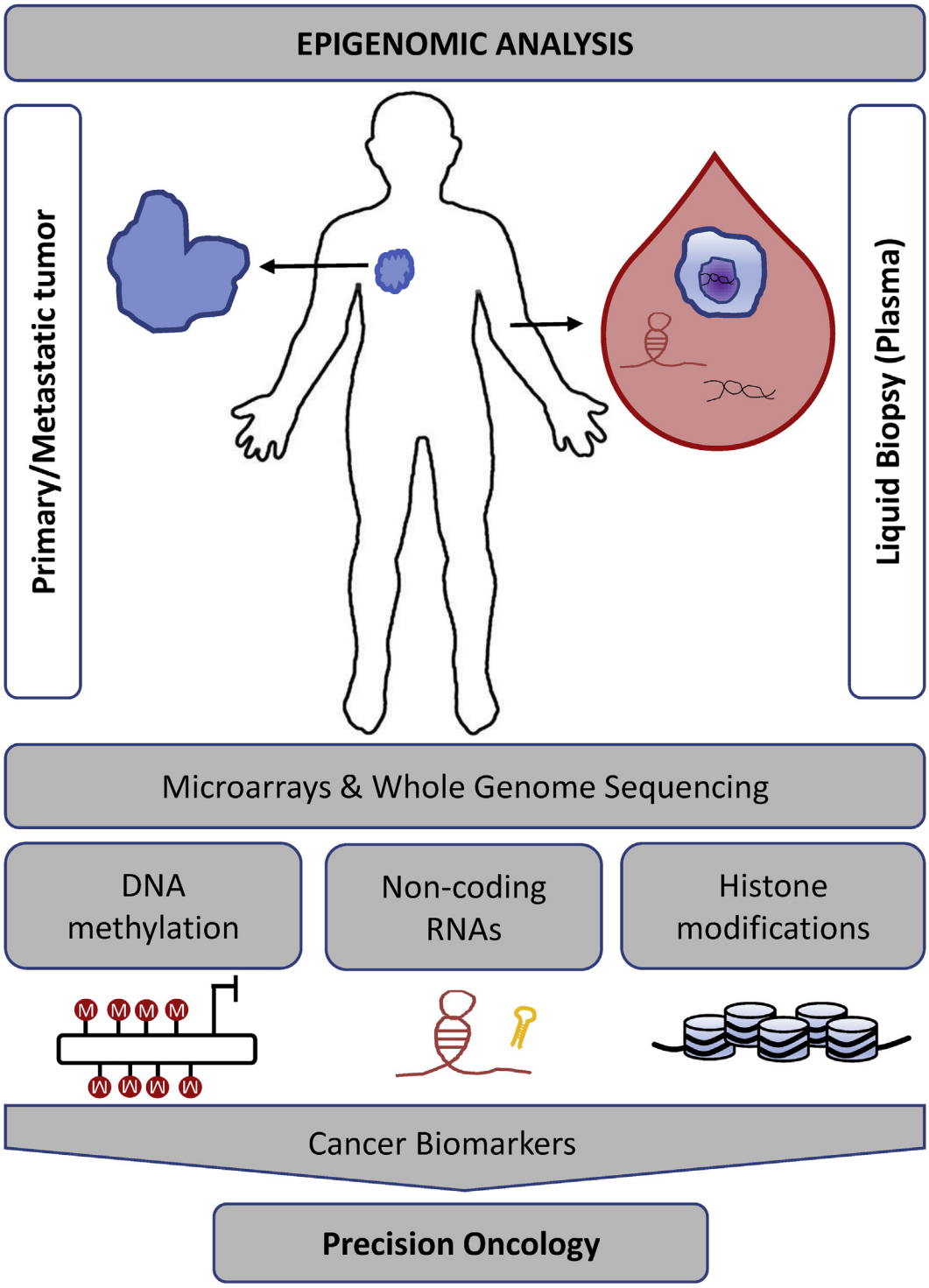

Fig 1. Epigenomic analysis for precision oncology biomarkers. Microarray systems and whole generation sequencing are emerging epigenomic tools that lead to genome-wide profiling of epigenetic modifications (DNA methylation, noncoding RNAs, and histone modifications) in primary or metastatic tumor samples or in liquid biopsies (plasma and other fluids). These approaches have found to be successful in identifying epigenetic cancer biomarkers with clinical utility for precision oncology.

sequencing (ChIP-seq) remains the standard for large-scale profiling of binding site locations for individual proteins and histone modifications. Typically, large numbers of cells (~10 million) are required for ChIP-seq. However, some methods have been recently developed (Nano-ChIP-seq, LinDA) to address this problem through post-ChIP DNA amplification.[57,58] In addition, chromatin interaction analysis with paired-end tag (ChIA-PET) is an emerging technology that has unique advantages in chromatin interaction analysis, and thus provides an insight into the study of transcription regulation. This method helps to generate high-resolution data of those genes that are being targeted by DNA bound proteins and regions with specific histone modifications.[59]

Another layer of complexity in cancer epigenomics is the analysis of the expression of different types of ncRNAs (microRNAs, lncRNAs, and others). ncRNAs can be identified in numerous types of clinical samples such as archived tissues and plasma by means of methods such as microarray platforms and NGS. These methodologies are based on different principles, since NGS involves an amplification of ncRNAs step, whereas microarrays are based on hybridization of specific probes. Although both systems have shown sufficient sensitivity and specificity to detect ncRNAs as biomarkers, the overall choice of the most appropriate method for future studies should take into account the amount of specimen available, the number of samples analyzed, and the costs associated with each analytical platform. If the aim is to detect novel ncRNAs, then the preferred choice of platform is likely to be NGS, because it is able to deliver the greatest amount of data and requires no prior knowledge of the sequences to be identified.[60,61]

SMOKING-RELATED EPIGENETIC REGULATION

Environmental factors are considered as strong determinants for epigenetic regulation. Among these factors, cigarette smoking, a major preventable public health problem that is associated with pulmonary disease, is an established critical factor for epigenetic modification. DNA methylation, histone modifications, and expression regulation of noncoding RNA have all been shown to be altered by tobacco smoke.

A recent study identified a set of genes that are differentially methylated in the parenchymal tissue of smokers as compared with nonsmokers.[62] Moreover, a systematic review of DNA methylation studies identified 17 publications addressing the association of active smoking exposure with methylation modifications in blood DNA, including 14 recent epigenome-wide association studies.[63] This systematic review revealed the existence of smoking-related differentially methylated CpG sites in whole blood samples that reflect not only current but also lifetime or long-term exposure to active smoking.[63] This influence of smoking on DNA methylation patterns can determine the susceptibility to smoking-related disease development. Of note, aberrant methylation of proto-oncogenes and tumor suppressor genes can already be detected in the smoking-damaged bronchial epithelium of cancer-free heavy smokers, suggesting that aberrant methylation might be an ideal candidate biomarker for lung cancer risk assessment and the monitoring of response to chemopreventive agents.[64]

Similarly, earlier reports have demonstrated the cigarette smoke–induced remodeling of chromatin by hyperacetylation of histone and decreased HDAC activity in lungs of smokers.[65] Histone marks in histone H3 and histone H4 (including lysine acetylation, lysine methylation, and arginine methylation [eg, H4K31me2 and H4R35me2]) have been identified as playing an important role in the epigenetic state during the pathogenesis of smoking-induced chronic lung diseases, such as chronic obstructive pulmonary disease and lung cancer.[66]

The effect of cigarette smoke on histone modifications and DNA methylation appears to be mediated in part by its ability to induce oxidative stress and inflammation.[67] This pro-oxidant and proinflammatory microenvironment created by cigarette smoke is also able to regulate the expression of noncoding RNA.[68]

Efforts have been made to compare the microRNA profile of normal lung epithelial cells in the presence/absence of cigarette smoke condensate (CSC) exposure. Some of the microRNA's modified by this condition are *miR-31*, *miR-200b*, *miR-200c*, *miR-205,* and *miR-487*. *miR-31* was found to be overexpressed after CSC exposure, both in normal human respiratory epithelia and lung cancer cells.[69] Moreover, samples obtained from lung cancer patients showed higher *miR-31* expression levels compared with paired adjacent normal tissue, and former smokers presented significantly higher levels of this microRNA compared with never-smokers. miR-31 was shown to have a protumoral effect in cells, promoting cell proliferation and tumor growth through direct targeting of *DDK-1* and *DACT-3*, antagonists of the *WNT* signaling pathway.[69] A similar example of microRNA regulated by CSC is miR-487b. Xi et al found that CSC exposure in normal human respiratory epithelia and lung cancer cells significantly reduces *miR-487b* expression, a tumor suppressor microRNA. This microRNA is regulated by promoter methylation and it has been shown to be relevant during pulmonary tumorigenesis, as some of its

direct targets are *SUZ12*, *BMI1*, *WNT5A*, *MYC,* and *KRAS*.[70]

Specific microRNAs have been related to an epithelial-to-mesenchymal transition (EMT) phenotype, a process also linked to E-cadherin loss after CSC exposure.[71] *miR-200b*, *miR-200c*, and *miR-205* presented a decreased expression due to hypermethylation in their promoters, after tobacco exposure.[72] Their downregulation induces both EMT and cancer stem cell phenotypes in normal human epithelial cells.[72]

Tobacco smoke has not only been shown to modify microRNAs expression in lung-derived cells in in vitro models but also in circulating microRNAs from patients. A comparative analysis of plasma microRNAs revealed significant deregulation of 35 microRNAs in healthy middle-aged smokers. Functional enrichment analysis showed that these microRNAs were related to the immune system and hormonal regulation.[73] Other studies using a similar approach identified elevated levels of *miR-124*,[74] and decrease in *miR-223* as well as increases in *miR-29b* and *RNU-6*[75] in smokers, in comparison with nonsmokers. Takahashi et al.[76] described that a larger number of microRNAs were found in the plasma of healthy smokers than in nonsmokers and that two-thirds of them (43 microRNAs) were upregulated. Many of these microRNAs were previously reported as potential biomarkers of disease and quitting smoking reversed the altered patterns of expression, which returned to resembling those of nonsmokers.

Expression of several lncRNAs has also been shown to be altered in lung epithelial cells as a consequence of CSC exposure. Acute or chronic addition of cigarette smoke extract to human epithelial bronchial cells showed a positive feedback loop that involved lncRNA *CCAT1*, which in turn upregulated Myc via repression of *Let-7c*.[77] This mechanism seems to promote the malignant transformation of these cells. *CCAT1* also inhibits *miR-218* on CSC exposure, which results in an altered cell cycle through *BMI1* and suggests a mechanism for lung cancer development.[78] Other examples that reveal changes in lncRNAs as a consequence of cigarette smoke extracts are *MALAT1* and *HOTAIR*.[68] *HOTAIR* expression induced by CSC causes EMT, and the use of a siRNA targeting this lncRNA reverses the EMT phenotype and the malignancy of cells.[79]

Therefore, smoking-related epigenetic marks have been proposed as suitable biomarkers for predicting the susceptibility to lung diseases. However, due to the strong influence of smoking on epigenetic modifications, the smoking status of patients should be taken into account as a confounder in analyses when the aim of the study is to identify specific epigenetic biomarkers of diseases such as lung cancer.

EPIGENOMIC BIOMARKERS FOR PRECISION ONCOLOGY

Cancer biomarkers play an important role in clinical practice due to their contribution to the improvement of disease stratification for diagnosis, drug target discovery, evaluation of exceptional responders, and the study of the mechanisms of acquired resistance.[80] The emerging paradigm of precision oncology seeks to determine tumor-driving networks and biomarkers that function in a particular patient's tumor, and then design a rational combination therapy.[81] Hopefully, this strategy will facilitate medical prevention and optimal therapy selection, thus improving the patient's quality of life and reducing overall cost for the public healthcare system. Over the last decade, there has been increasing interest in cancer biomarkers due to the rapid development in high-throughput technologies, involving screening of cancer-epigenomic features at a genome-wide scale using microarrays and whole genome sequencing. Several multiomic studies have been carried out in recent years leading to the identification of potential epigenetic biomarkers with clinical use (Fig 2).

DNA methylation constitutes an important epigenetic layer of transcriptional and regulatory control that has emerged as the most promising biomarker in the epigenomic field. Using a genome-wide DNA methylation approach, we have identified a novel epigenetic signature for prognosis in early-stage NSCLC patients.[82] Moreover, another study obtained a 3-gene methylation panel with diagnostic value in the same type of tumor.[83] Although DNA methylation marks have usually focused on classical coding genes, there is also an interplay between this epigenetic mechanism and noncoding RNAs. Thus, there are several epigenomic studies that have found DNA methylation–associated silencing of noncoding RNAs.[47,84] This new point of view can be now addressed with the current development of epigenomic tools that could lead to the identification of new epigenetic disrupted noncoding RNAs as biomarkers in lung cancer. In summary, profiling DNA methylation across the genome is critical to understanding the influence of epigenetics in normal biology and disease. Moreover, the development of this kind of epigenomic-based strategies and assays holds great promise and could guide more precise therapies associated with better outcomes for cancer patients.

CLINICAL UTILITY OF EPIGENETIC ANALYSIS IN LIQUID BIOPSIES

Epigenetic biomarkers in liquid biopsy may serve as surrogate markers for a patient's follow-up and early detection of tumor relapse (Fig 2). The number of

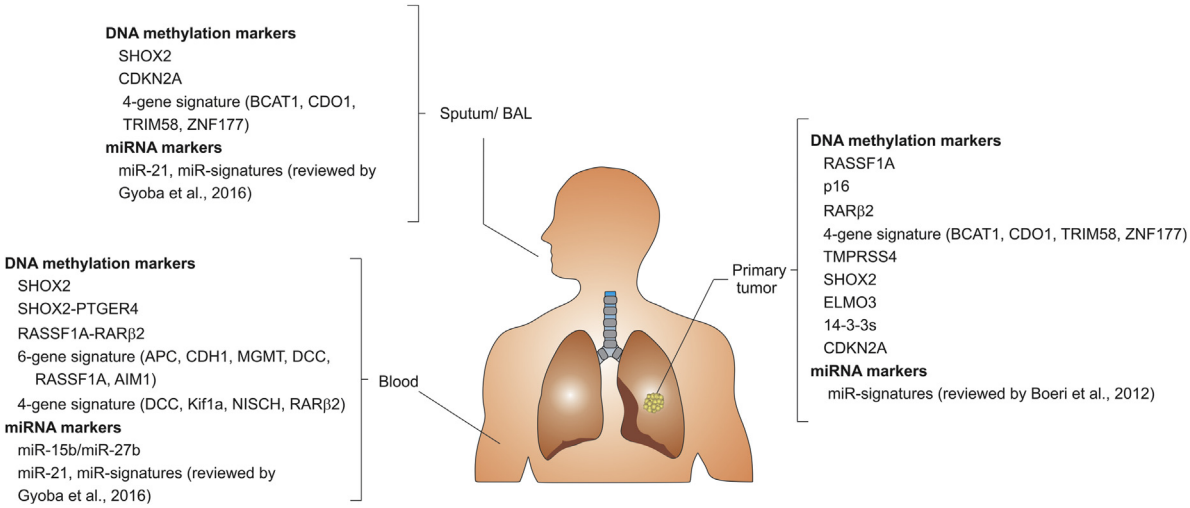

Fig 2. Epigenetic biomarkers in lung cancer. Selected epigenetic markers with relevance in lung cancer in primary tumors or liquid biopsy (sputum, bronchoalveolar lavage (BAL), and blood).

studies focusing on epigenetic biomarkers in liquid biopsy is still limited, but some of these markers have already reached the clinical scenario and others are currently in the process of validation. In 2010, Schmidt *et al.* showed that the DNA methylation status of *SHOX2* could be used for the diagnosis of lung cancer in bronchial aspirates.[85,86] Subsequently, *SHOX2* methylation was studied in more than 400 plasma samples and was found to identify lung cancer patients with 60% sensitivity and 90% specificity.[87] In both studies, this marker showed higher sensitivity for advanced patients (up to 80%–90%), as well as for squamous cell carcinoma histology. A commercial kit for the detection of *SHOX2* is available under the name Epi proLung.

The study of more than one marker has been used as a strategy to overcome low sensitivity. Plasma detection of both *SHOX2* and *PTGER4* combined was found to be an efficient way to discriminate between malignant and nonmalignant lung disease (AUC = 91%–98%).[88] In line with this, we have recently identified, using a multivariate logistic regression model, a 4-gene signature (*BCAT1*, *CDO1*, *TRIM58*, and *ZNF177*) with higher diagnostic efficacy than conventional cytology in minimally-invasive bronchial aspirate samples.[48] Tumor suppressor genes such as *RARB2* and *RASSF1A* genes were differentially methylated in normal and tumor blood samples (87% sensitivity and 75% specificity).[89] A panel of 4 hypermethylated gene promoters (*DDC*, *KIF1A*, *NISCH,* and *RARB*) was found when plasma samples from NSCLC patients were compared with controls.[90] Real-time PCR analysis found methylation of at least 1 gene in 73% of patients, with a sensitivity of 73% and specificity of 71%. Sidransky et al analyzed a panel of 6 tumor suppression genes (*APC*, *AIM1*, *CDH1*, *DCC*, *MGMT,* and *RASSF1A*) using cell-free serum DNA in NSCLC.[91] In 84% of patients at least one of the genes was methylated. The authors showed that genes analyzed independently performed worse than when combined.

The diagnostic potential of DNA methylation has also been demonstrated in other cancer types. *GSTP1* methylation is the most relevant DNA marker in prostate cancer (PCa) and different studies have found abnormal promoter hypermethylation in 80%–90% of PCa patients.[2] *GSTP1* methylation has been detected in blood and urine[92,93] and has been suggested as a complementary test to improve the diagnostic accuracy of PSA in PCa.[94] In CRC, hypermethylation of *APC*, *MGMT*, *RASSF2,* and *WIF1* in plasma showed 87% sensitivity and 92% specificity.[95] The diagnostic potential of *TMEFF2*, *NGFR,* and *SEPTIN 9* hypermethylation in blood samples from CRC patients has also been demonstrated.[96] Recently, the combination of *SEPTIN9* and *SHOX2* DNA methylation status has been shown to be useful for diagnosis in CRC,[97] as well as to differentiate between benign and malignant pleural effusions.[98] Several commercial kits for CRC detection in noninvasive samples based on DNA methylation analysis are currently available, including Epi proColon (*SEPTIN9*), ColoSure (*vimentin*), and Cologuard (a test that combines the analysis of *KRAS* mutations with *NDRG4* and *BMP3* methylation). With the implementation and standardization of novel ultrasensitive techniques for detection of DNA methylation changes, such as droplet digital PCR (ddPCR), it is expected that accuracy in the detection of these markers in liquid biopsies will be significantly increased. ddPCR

provides the precise number of methylated/unmethylated copies and also offers the opportunity to perform quantitative multiplex analysis of a panel of different target genes.[99]

miRNAs have also been used in liquid biopsy for diagnosis, patient follow-up, and prediction of response to therapies, in NSCLC and in other malignancies. Circulating miRNAs are stable in body fluids and can be quantified by qRT-PCR in a relatively easy and cost-effective way. In fluids, miRNAs can be found in association with lipoproteins, RNA-binding proteins, and inside exosomes, where they are protected from degradation by RNAses.[100] Focusing on the information available in lung cancer, miRNA profiles for diagnosis and prognosis have been reported in whole blood, serum, plasma, and sputum. In sputum, the sensitivity of miRNA panels varies between 61% and 100%, and specificity is within the range of 80%–100%. *miR-210* has been the most widely reported miRNA in sputum, and *miR-21*, *miR-31*, and *miR-155* have also been described in several papers.[101] From the analyses carried out in serum, the sensitivity/specificity values are encouraging, but there is a surprising lack of overlap between these studies. Hennessey et al. have described that the combined analysis of *miR-15b* and *miR-27b* can detect lung cancer with 100% sensitivity and 84% specificity.[102] Other studies have also shown excellent diagnostic performances using different sets of miRNAs.[103-105] Regarding plasma, *miR-21* has been identified as a lung cancer biomarker in several studies.[101] In addition, a study by Sozzi et al examining a miRNA signature classifier together with computerized axial tomography has reported the utility of this miRNA panel in NSCLC screening programs.[106]

Considering all the studies performed in liquid biospies in lung cancer, *miR-21* seems to be a robust candidate, but panels that include several miRNA are likely to be more accurate than a single miRNA.[107] Nonetheless, the lack of consistency between the different studies shows that identification of consistently deregulated miRNAs in liquid biopsies is not an easy task. For the clinical implementation of miRNAs as biomarkers, it seems clear that reproducibility, robustness, and extensive validation in different cohorts of patients will have to be further pursued in future studies.

In recent years, there has been increasing interest in the evaluation of circulating lncRNAs as diagnostic/prognostic markers in fluids. Of particular importance is the lncRNA prostate cancer antigen-3 gene (*PCA3*, also called *DD3*), the most widely investigated lncRNA as a diagnostic tool. This marker is overexpressed in 50%–90% of prostate cancer cases.[108] *PCA3* can be quantified in urine with the PROGENSA *PCA3* test.[109] Other lncRNAs that are being used as potential biomarkers in liquid biopsies include *MALAT-1* for prostate cancer, *UCA1* for bladder cancer, *RP11-445H22.4* for breast cancer, and *ANRIL* for lung cancer. Increased levels of circulating *SPRY4-IT1*, *ANRIL*, and *NEAT1* have been reported in NSCLC.[110] Apart from *PCA3*, the potential utility of these biomarkers is again promising although they may be far from being of practical use in a clinical setting at this point.

Future studies using epigenetic biomarkers in liquid biopsies should address the following issues: (1) validation in independent cohorts of patients; (2) determination of the most accurate technology to analyze the biomarkers and standardization of the methodology; (3) concordance between levels in liquid biopsy and tumor and whether or not those particular biomarkers may represent tumor heterogeneity; and (4) analysis during cancer progression and after therapy.

THERAPIES BASED ON EPIGENETIC MODIFICATIONS

With the aim of reversing the epigenetic alterations that trigger cancer-specific events, several therapies have been developed based on untargeted and targeted strategies (Fig 3). In untargeted approaches, the catalytic activity of the enzyme responsible for the epigenetic modification is blocked, and consequently the effect spreads throughout the genome. Currently, this group is primary represented by DNA methylation inhibitors and HDAC inhibitors.[111,112] In contrast, in targeted approaches, direct epigenetic modifications are made to specific loci in the genome with a local effect. For this, fusion proteins consisting of a DNA-binding domain providing locus specificity and an effector domain facilitating the chemical modification are required.[113] As will be described below, locus specificity is currently achieved with zinc finger proteins (ZFP), transcription activation-like effector proteins (TALEs), and clustered, regularly-interspaced short palindromic repeats-associated protein 9 (CRISPR-Cas9).[113] Furthermore, the effector domain can be represented either by different enzymes (DNMT, HMT, HDAC, etc)[114] or by transcription activators or repressors, such as the viral protein VP64 or super KRAB domain.[115,116]

Untargeted therapies: DNA methylation and HDAC inhibitors. ***DNA methylation inhibitors.*** Considering the common finding of TSG promoter hypermethylation in cancer cells and tumor biopsies, several strategies targeting the methylation machinery have been developed with the aim of restoring TSG gene expression and preventing tumor proliferation.[117] Currently, this therapeutic approach is represented by the hypomethylating agents (HMAs) 5-azacytidine and 2'-deoxy-5-azacytidine. Once incorporated into nucleic acids, these chemical analogs of cytidine covalently and irreversibly

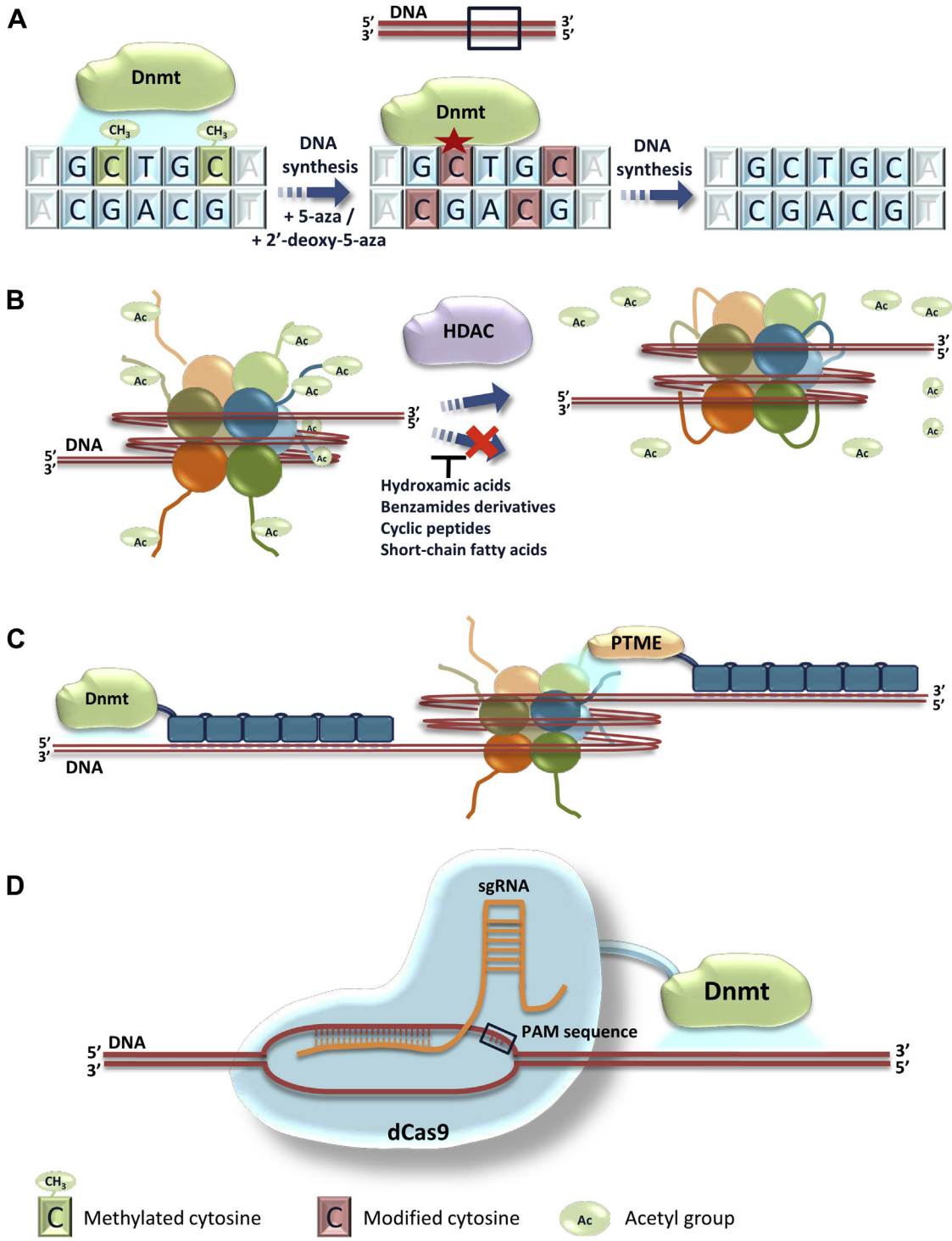

Fig 3. Untargeted and targeted approaches for epigenetic editing. **A,** Methylation at cytosines in DNA is achieved by the action of Dnmt enzymes (eg, *Dnmt3a*). This renders a compacted chromatin structure and a repressed transcriptional state. Through several cell divisions, cytosine analogs such as 5-azacytidine and/or 2'-deoxy-5-azacytidine are incorporated across the genome, thus covalently and irreversibly binding to Dnmts. As a consequence, active Dnmts enzymes are depleted in cells, and hypomethylation takes place throughout the genome. **B,** Acetylation at histone cues is associated with a relaxed chromatin structure and gene expression. Several compounds belonging to 4 different chemical groups (eg, hydroxamic acids, benzamide derivatives, cyclic peptides, and short-chain fatty acids) can inhibit the action of HDAC and prevent transcriptional repression associated with

bind to DNMTs, causing global DNA hypomethylation and DNA damage induction.[118,119]

The first successful clinical application of an untargeted epigenetic therapy was achieved in hematological tumors. As a consequence, both 5-azacytidine and 2'-deoxy-5-azacytidine were approved by the FDA and the EMA (as Vidaza and Decitabine or Dacogen, respectively) for the treatment of acute myelogenous leukemia, the most prevalent acute leukemia in adults characterized by an epigenetic dysfunction.[120] Importantly, these compounds are currently the first therapeutic choice for patients with myelodisplasic syndromes who are not candidates for allogenic hematopoietic stem cell transplantation.[121] Furthermore, several clinical studies are expanding the curative possibilities of DNMTIs to also include solid malignancies. Two clinical trials are testing azacitidine (NCT01193517) and decitabine (NCT02316028) in patients with metastatic CRC, for which no curative treatment option exists. In addition, an ongoing phase II clinical trial (NCT02795923) is evaluating Nivolumab (an immune checkpoint inhibitor) alone or in combination with oral decitabine/tetrahydrouridine (THU) as second-line therapy for NSCLC. It is expected that blocking DNA methylation in lung tumor cells will act synergistically with Nivolumab improving the objective response rates of the immune checkpoint inhibitor alone. The rationale for the use of THU is its inhibitory activity on cytidine deaminase, a highly expressed enzyme in solid tissues of the body, which breaks down decitabine to its uridine metabolites, thus reducing its bioavailability.[122] THU is also being tested in other clinical trials for head, neck, lung, and bladder neoplasms in combination with decitabine analogs (NCT00978250) and also in sickle cell disease (NCT01685515). Although not yet tested in clinical trials, other compounds with DNMTI activity have been developed. Zebularine (1-(β-D-ribofuranosyl)-1,2-dihydropyrimidin-2-one), a cytidine analog that displays higher stability and less toxicity than the aforementioned Vidaza and Dacogen, has concomitant inhibitory activity towards cytidine deaminase.[123] This compound has been found to inhibit the growth of A549 lung cancer cells (via cycle arrest and apoptosis[124]) and also of hepatic carcinoma HepG2 cells.[125] In addition, non-nucleoside DNMTIs such as RG108, which targets the catalytic center of *DNMT1*, have demonstrated antitumoral effect in prostate cancer cell lines. Importantly, as these molecules are not nucleoside analogs, they are not incorporated into DNA, thus potentially displaying reduced toxicity.[126]

Based on the successful outcomes in hematological malignancies, new opportunities have emerged for the treatment of distinct types of solid malignancies with HMAs. Nevertheless, further research endeavors are warranted to optimize drug dosage and scheduling of the agents currently available and to potentiate their place in cancer therapeutics either alone or as coadjuvant therapies.

HISTONE DEACETYLASE INHIBITORS

In a cancer context, acetylation homeostasis is shifted toward deacetylation, and repression of regulatory genes increases migration, invasion, and angiogenesis in normal cells, leading to the origin and progression of cancer.[23] Consequently, great research efforts are being directed toward developing inhibitory molecules for HDAC enzymes as therapeutic approaches in several malignancies.[117]

Molecules employed as inhibitors of HDAC activity (HDACI) can be grouped into 4 different classes, depending on their chemical properties: hydroxamic acids, benzamide derivatives, cyclic peptides, and short-chain fatty acids.[111] Of these, 2 compounds belonging to the hydroxamic acid group (vorinostat and belinostat) and a cyclic peptide (romidepsin) have recently been approved by the FDA for the treatment of cutaneous/peripheral T-cell lymphoma.[127-129] In addition, ongoing clinical trials are testing these HDACI in combination with other chemotherapy agents for the treatment of other disorders. For instance, vorinostat is being tested as a coadjuvant for the treatment of advanced solid malignancies (NCT02042989, NCT01023737), oropharyngeal squamous cell carcinoma (NCT01064921), multiform glioblastoma (NCT00555399), and NSCLC (NCT01064921). Importantly, Juergens et al have reported that the combination of entinostat, a benzamide derivative, with a low-dosage of azacitidine

deacetylation and a more compacted chromatin structure. Histone 1 is not represented. **C,** Zinc finger proteins are composed of an array of modules, each one recognizing 3 nucleotides in the DNA sequence. Engineered Zinc finger proteins fused to different effectors (eg, Dnmt, post-transcriptional modification enzymes) can target specific loci in the genome to add distinct chemical modifications (eg, methylation on DNA or covalent modification on histone cues). **D,** dCas9 nucleases recognize a specific locus in the DNA based on Watson-Crick base pairing between a designed sgRNA and the genomic region of interest. An effector domain (eg, *Dnmt3a*) introduces the desired chemical modification; in this case, cytosine methylation. Dnmt, DNA methyl transferase 3a; HDAC, histone deacetylase; Ac, acetyl group; PTME, post-transcriptional modification enzymes; dCas9, deficient CRISPR-associated nuclease 9; sgRNA, single-guide RNA; PAM, protospacer adjacent motif.

resulted in objective and durable responses in patients with refractory recurrent metastatic NSCLC. In addition, this combined epigenetic therapy led to demethylation of 4 silenced genes, which could be assessed in plasma samples and correlated with improved progression-free and overall survival.[130]

Encouraged by the aforementioned achievements, newly developed HDACI compounds are broadening the therapeutic options for different malignancies. Alzoubi et al. demonstrated that the combined treatment of suberoylanilide hydroxamic acid and 5-fluorouracil and oxaliplatin induced mitotic cell death in multidrug-resistant CRC HT-29 cells.[131] In NSCLC, treatment with givinostat (ITF2357) in sequential combination with pemetrexed induced histone acetylation and inhibited cell growth, viability, and apoptosis in vitro and also increased cell survival in mice models.[132] In another preclinical study, the newly developed ST3595 hydroxamic acid derivate inhibited the invasive properties of H460 and A549 NSCLC cells more than vorinostat, through a mechanism involving the upregulation of the antimetastatic gene KiSS1.[133] Finally, entinostat, a benzamide acting as an *HDAC1* and *HDAC3* inhibitor, has successfully passed phase I/II clinical trials for the treatment of NSCLC[134] although it still requires further validation in phase III studies to become a reality in clinical practice. Remarkably, since HDAC are not histone-specific enzymes,[135] HDACI not only influence chromatin structure but spread their effect at multiple cellular levels as nonhistone proteins.[111]

Targeted therapy: Zinc finger and CRISPR/Cas9 technologies. Targeted epigenetic editing field is an incipient area of research, less well developed than genetic editing from which epigenetic tools derive. To our knowledge, no single publication has reported specific achievements in lung cancer to date. However, some successful studies that anticipate the clinical application of targeted epigenetic editing are worthy of mention. With regard to transcription activator–like effectors, several inherent limitations (such as susceptibility to DNA rearrangements, their large size, and sensitivity to methylated DNA)[114] have reduced their use in favor of other alternatives (ZFP and CRISPR-Cas9) and therefore they will not be reviewed in the present paper.

ZINC FINGER PROTEINS

Zinc finger proteins are DNA-binding domains commonly found in eukaryotic transcription factors, where each module of 30 aminoacids in a beta-beta-alpha structure recognizes 3 bp of the DNA sequence. Therefore, assembling 6 or more ZFP modules in tandem repeats allows unique specificity throughout the genome.[113,136]

Rivenbark et al. provided a brilliant example in cancer research by engineering zinc finger motifs fused to *DNMT3a* targeting and silencing the expression of the tumor suppressor gene Maspin and the oncogene *SOX2* in poorly tumorigenic SUM159 and MCF7 breast cancer cells, reversing their phenotype.[137] Nunna et al transfected SOKV3 ovarian cancer cells with a chimeric ZF-Dnmt3a C terminal catalytic domain protein directed at the epithelial cell adhesion molecule (EpCAM) promoter.[138] EpCAM is overexpressed in several epithelial cancers, including ovarian, breast, pancreatic, urothelial, and gallbladder carcinoma and its expression levels inversely correlate with survival of patients.[138,139] Falahi et al. were able to repress the expression of the human epidermal growth factor receptor-2 (*HER2/neu*) in ovarian and breast cancer cells by specifically methylating its promoter by means of a ZFP fused to the HMT G9a and SUV39-H1.[114] HER2/neu is overexpressed in several cancers (eg, breast, gastric, and ovarian cancers) and its upregulation is associated with poor prognosis, due to its role in regulating cell proliferation.

Aberrant angiogenesis occurs in different pathologic conditions and is a hallmark of cancer.[140,141] In this respect and by employing an engineered ZF-Dnmt3a-Dnmt3L, Siddique et al. successfully silenced the expression of vascular endothelial growth factor A (*VEGF-A*), the main regulator of angiogenesis, in an ovarian cancer SKOV3 cell line by methylating 12 CpGs at the *VEGF-A* promoter.[142] Similarly, Snowden et al decreased the expression of *VEGF-A* in HEK293 cells by specifically methylating H3K9 with specific ZF-SUV39H1/G9A constructs.[143]

The aforementioned studies exemplify the potential of ZFP for directing desired modifying enzymes to specific genomic loci. Nevertheless, the introduction of CRISPR-dCas9 technologies for epigenome editing has represented a revolution in the field, in part because of an easier design and the higher specificity of a RNA-DNA–based interaction, rather than the protein-DNA–based interaction provided by ZFP.

CRISPR/CAS9

CRISPR-Cas9 is considered an acquired immune system in bacteria and archaea.[144] These prokaryotes incorporate multiple foreign DNA sequences in a hypervariable region of its genome, the CRISPR locus. Subsequently, Cas nucleases incorporate cleaved transcripts from CRISPR loci and then scan the cytoplasm searching for foreign complementary sequences to be cleaved, in a RNAi-like mechanism of action.[145] For epigenetic editing purposes, *Cas9* proteins engineered to be deficient in nuclease activity (dCas9) can be fused

to a myriad of effector domains, expanding the epigenome editing tool repertoire. The mechanism of action is based on a short-guide RNA (sgRNA) complementary to the target sequence directing the complex to the appropriate loci and requiring only an appropriate protospacer adjacent motif (PAM) sequence (eg, 5′-NGG-3′ for the *Streptococcus pyogenes* dCas9) at the 3'-end of the targeted sequence for efficient binding and cleavage.[113]

As a proof of principle, Hilton et al demonstrated that programmed dCas9 fused to the H3K27-acetylytansferase *p300* is a valid tool to promote gene expression throughout the genome both from promoters and from proximal and distal enhancers, as demonstrated with *IL1RN*, *MYOD1,* and *OCT4* genes.[146] In another interesting approach, Thakore et al targeted dCas9-KRAB to a distal HS2 enhancer, which regulates the expression of multiple globin genes. Expression of *HBE1*, *HBG1/2*, and *HBB* was reduced with minimal off-target effects.[147] Notably, *KRAB* has no catalytic activity but, instead, recruits proteins to assemble a heterochromatin-forming complex that represses gene expression through histone methylation and deacetylation.

Vojta et al., published a seminal study in which methylation at specific promoters was achieved by using dCas9 fused to the catalytic domain of *DNMT3a*.[148] They specifically methylated and downregulated the expression of both *BACH2* and *IL6ST* genes, which are involved in autoimmune diseases such as inflammatory bowel disease. Shortly after, Stepper et al achieved widespread methylation at the *EpCAM*, *CXCR4,* and *TFRC* gene promoters both in HEK293 and ovarian cancer SKOV3 cells.[149] To do so, authors employed and engineered a dCas9-Dnmt3a-Dnmt3L construct. In addition, methylation marks can also be removed with this engineered system, but in this case by tethering a *TET-1* DNA demethylase enzyme to the dCas9 protein.[150,151]

Although CRISPR-dCas9 technology for epigenome editing is only taking its initial steps, the ease and robustness in targeting desired sequences, with tenuous off-target effects, assures great success in the epigenetic editing arena. In addition, this tool is multiplexable and several gRNAs can act in concordance to target the same or different genes at the same time.[152] In addition, as *Cas9* derived from different prokaryotes require different protospacer adjacent motif sequences, the combination of different *Cas9* can broaden the spectrum of target genes.[113]

CONCLUSIONS AND FUTURE PERSPECTIVES

Knowledge of epigenetic modifications in cancer has increased exponentially in recent years thanks to the application of novel high-throughput technologies. This kind of "omic" approach, referred to as epigenomic, is revolutionizing our understanding of how cancer develops and is providing new avenues for detection, monitoring, and therapy. Epigenetic signatures will also define in a more accurate way cancer subtypes, prognosis, response to chemotherapy and epigenetic drugs, and will help the implementation of personalized medicine. Epigenetic modifications are common events during cancer progression and metastasis in hematological malignancies and solid tumors and, therefore, they can be used as biomarkers and targets with clinical use. In NSCLC, a deadly cancer which has been the focus of our review, investigation of epigenetic/epigenomic modifications has provided a variety of biomarkers (hyper/hypomethylated DNA gene promoters, histone PTMs, miRNAs and lncRNAs) that allow early detection and follow-up of patients. One example that can be currently used as a commercial kit is *SHOX2*. With the establishment of the epigenomic era it is expected that the identification of novel epigenetic signatures can be translated into clinical practice. Moreover, the therapy targeting epigenetic pathways is currently being applied in hemato-oncology and is being tested in clinical trials for solid tumors. As some of the drugs are nonspecific because they affect numerous genes/transcripts of the epigenetic machinery, it may be necessary to design novel targeted strategies instead of global processes. In this sense, zinc finger proteins– and CRISPR/Cas-9–based approaches may offer a solution, although such therapies are still in their very early stages of development.

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REVIEW

MicroRNAs: New players in endometriosis

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Abstract

Endometriosis is an estrogen-dependent inflammatory disorder that limits the quality of life of affected women. This pathology affects 10% of reproductive-age women, although the prevalence in those patients experiencing pain, infertility or both is as high as 35%-50%. Endometriosis is characterized by endometrial-like tissue outside the uterus, primarily on the pelvic peritoneum, ovaries and the pouch of Douglas. Despite extensive research endeavours, a unifying theory regarding the exact etiopathogenic mechanism of this high prevalent and incapacitating condition is still lacking, although it has been suggested that epigenetics could be involved. MicroRNAs (miRNAs), one of the epigenetic players, are small non-coding RNAs that can act as post-transcriptional regulators of gene expression, reducing the expression of their target mRNAs either inhibiting its translation or promoting its degradation. MiRNA expression profiles are specific of tissue and cell type. Abnormal miRNA expression has been described in different pathological conditions, such as a myriad of oncological, cardiovascular and inflammatory diseases and gynecological pathologies. In endometriosis, miRNA expression patterns of eutopic endometrium from patients and control women and from different endometriotic lesions have been described. These small non-coding molecules have become attractive candidates as novel biomarkers for an early non-invasive diagnosis of the disease, which could suppose a valuable benefit to the patients in terms of improvement of prognosis and reduction of the ratio of recurrence. In this systematic review we will focus on the role of miRNAs in the pathophisiology of endometriosis.

Key words: MicroRNAs; Endometriosis; Epigenetics;

Angiogenesis; Biomarkers

Core tip: Endometriosis is an estrogen-dependent inflammatory disorder that limits the quality of life of affected women. Nowadays, a unifying theory regarding its exact etiopathogenic mechanism has not been achieved yet. Our objective is to review the current literature to better understand the role of microRNAs, one of the epigenetic players, in the pathophisiology of endometriosis and their potential as novel diagnostic biomarkers to guide therapeutic interventions in endometriosis.

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INTRODUCTION

Endometriosis is an estrogen-dependent inflammatory disorder that limits the quality of life of affected women[1-3]. This pathology affects 10% of reproductive-age women, although the prevalence in those patients experiencing pain, infertility or both is as high as 35%-50%[4]. The prevalence of this condition is estimated around 176 million worldwide, with an average diagnostic delay of 7 years[5], being the mean age at diagnose 32.5-36.4 years, depending of the study population[5,6].

Endometriosis is characterized by endometrial-like tissue outside the uterus, primarily on the pelvic peritoneum, ovaries and the pouch of Douglas. These extrauterine lesions are responsible for the main symptoms, pelvic pain and infertility[1].

Despite extensive research endeavours, a unifying theory regarding the exact etiopathogenic mechanism of this high prevalent and incapacitating condition is still lacking. Several authors have reported a hormonal, immunity and a genetic base for this gynecological disorder. However, a growing body of evidence suggests that epigenetics could also be involved[7], with an exponential increase of papers published on this issue in recent years.

Epigenetics refers to the study of mechanisms that control gene expression in a potentially heritable way without affecting DNA sequence. MicroRNAs (miRNAs), DNA methylation and modifications of the chromatin structure represent the different types of the known epigenetic modifications, exerting their regulatory effect additively[8]. In this review we will focus on the role of miRNAs in the pathophisiology of endometriosis.

MiRNAs are small (19-22nt) non-coding RNAs that can act as post-transcriptional regulators of gene expression, reducing the expression of their target mRNAs either inhibiting its translation or promoting its degradation. MiRNAs usually regulate gene expression by binding to the 3' UTR (Untranslated Region) of their target mRNA. Importantly, several miRNAs can target a given mRNA and a single miRNA can target several mRNA, increasing the complexity of the regulatory mechanism mediated by these molecules[9-13]. In malignancies, miRNAs can act as oncogenes or tumor suppressors, depending on their targets[14-16]. It is important to highlight that the miRNA expression profiles are specific of tissue and cell type[9]. To date, more than 1881 miRNA precursors, coding for more than 2500 mature miRNAs have been described in humans[17].

MiRNAs were first described in 1993 by Lee *et al*[10] in the worm *Caenorhabditis elegans*. Since then, studies about biogenesis, functions, roles and characterisation of the mechanism of action of miRNAs have grown considerably and nowadays they are considered as excellent biomarkers of some diseases such as coronary artery disease[18-20], cancer[21,22], and several gynecological pathologies, including endometriosis[23,24].

PERITONEAL FACTORS AND ENDOMETRIOSIS

Endometriosis is a multifactorial disease in which endometrial and peritoneal factors such as those related to angiogenesis and proteolysis may be involved[25-27]. Peritoneal fluid (PF) is a complex suspension containing large amount of macrophages as well as endometrial and red blood cells, small molecules diffused from plasma through the mesothelial wall and other components dependent on ovarian contribution and local secretion such as steroid hormones and growth factors, respectively[28]. Because ectopic lesions located in the pelvic peritoneum are completely submerged in this fluid, their components have emerged as an important field of study[28-31].

It is well documented that endometriosis is characterized by an important inflammatory process[32-34] with and increased production of reactive oxygen species (ROS)[35-37]. Berkes *et al*[38] and Santulli *et al*[39] have identified significantly increased levels of protein oxidative stress markers in the PF from women with deep infiltrating endometriosis when compared with endometriosis-free controls. On the other hand, NETosis describes the mechanisms by which activated neutrophils expel their entire chromatin, serving as catch and kill scaffold against microorganisms, a structure designated as neutrophil extracellular traps (NETs). Furthermore, it is known that ROS are the major activator of NETosis. The involvement of NETosis in endometriosis was studied Berkes *et al*[38], who observed the presence of NET formation in virtually half of the patients with endometriosis, primarily in the

stage Ⅰ and Ⅱ group and rarely in controls, suggesting that NETosis is implicated in the initiation of the disease.

The contribution of immune system disorders to endometriosis has been proposed by several authors[2,40-42]. In this context, macrophage migration inhibitory factor (MIF) is arousing growing interest. MIF is a major pro-inflammatory factor found elevated in PF from women with endometriosis. Apart from its effect on activating and inhibiting macrophage mobility, it is also considered a critical upstream activator of innate immunity. MIF may be required for ectopic endometrial tissue growth and progression of endometriosis lesions *in vivo*[43]. Interestingly, miR-451 has been postulated to target MIF[44]. By using a murine model, Nothnick and coworkers[45] concluded that disruption of miR-451 expression in endometrial tissue impairs the ability of this tissue to establish ectopically. These authors also found elevated expression levels of miR-451 and diminished of MIF in ectopic endometriotic lesions (mainly peritoneal lesions) when compared with matched eutopic tissue. In addition, *in vitro* luciferase assays corroborated MIF as a target of miR-451 and forced expression of miR-451 reduced MIF and cell survival. Consequently, the aforementioned authors hypothesized that miR-451 over-expresses in ectopic lesions in an attempt to curtail endometriotic lesion/cell survival[46].

MIRNAS IN ENDOMETRIOSIS

Abnormal miRNA expression has been described in different gynecological pathologies, including malignancies[47-49], bening conditions as leiomyoma[50], adenomyosis[51], and endometriosis[11,52-54]. Among gynecological tumors, ovarian cancer represents the second most prevalent and the most lethal malignancy in developed countries[55,56], what could be explained by the difficulty of its diagnosis at early stages and the lack of effective treatments[49]. Recently reviewed by Davidson *et al*[57], miRNAs could be an invaluable tool to overcome the above mentioned limitations, regarding their potential role in diagnosis and progression of ovarian carcinoma as well as prediction of response to chemotherapy. For instance, miRNAs of the miR-200 family, the miR-199/14 cluster and the let-7 paralogs have emerged as potential therapeutic targets in ovarian cancer[49]. In addition, Lee *et al*[58] found that higher expression of miR-181d, miR-30c, miR-30d, and miR-30e-3p was associated with significantly better disease-free or overall survival in this condition. These both miR-30 and miR-200 families have also been associated with endometrial cancer, the most frequent gynecological malignancy[56,59]. In a recent work, Kong *et al*[59] reported miR-30c to be a tumor suppressor *via* the miR-30c-MTA-1 signaling pathway, with a decreased expression of this miRNA in tumor cells.

Regarding endometriosis, miRNA expression patterns of eutopic endometrium from control women and patients[53,60] and ectopic lesions from patients have also been described[11,53,61]. Although endometriosis is a benign condition, it shares common mechanisms with tumors (*e.g.,* tissue invasion, inflammation, reduced apoptosis and aberrant angiogenesis)[55]. In this context, the relationship between endometriosis and ovarian cancer, specially endometrioid and clear cell ovarian carcinoma, has been long reviewed[62-66], but recent literature on this issue points that existing data is not enough to establish a doubtless causality[55].

Among the pioneering studies addressing the miRNA expression patterns in endometrial and endometriotic tissues was the work published by Burney *et al*[67]. Four endometrial samples from women with endometriosis and three from endometriosis-free women in the early secretore phase of the menstrual cycle were assessed for miRNA expression by means of microarray analysis. After real time quantitative polymerase chain reaction (qRT-PCR) validation, the authors reported a downregulation of four miRNAs (miR-34c-5p, miR-34b*, miR-9 and miR-9*) belonging to two miRNA families (miR-34 and miR-9, respectively) in the eutopic endometrium of women with endometriosis compared to endometrium from control women. Notably, members of the miR-34 family mediate the p53-dependent suppression of proliferation[68]

Furthermore, Laudanski *et al*[60] conducted a study enrolling 25 endometriosis-free women and 21 patients with ovarian endometriosis in the proliferative phase in which the expression of 667 human miRNAs was examined. Validation of array results led to the corroboration that miR-483-5p, a regulator of IGF2, and miR-629-3p, involved in inflammation, were downregulated in the eutopic endometrium of patients in comparison to controls. The authors pointed to the idea that dysregulation of these genes could contribute to the overgrowth of endometrial tissue outside the uterus.

Human endometrium is a unique tissue that undergoes complex molecular, cellular, and functional changes on a cyclic basis under ovarian hormone regulation[69,70]. These changes are essential for uterine receptivity and can be grouped in three distinct phases: Proliferative, secretory and menstrual[71]. Thus, some authors hypothesized that miRNA expression could vary across the menstrual cycle[11,72]. For instance, Kuokkanen *et al*[72] showed that miRNA expression profiles of human endometrial epithelium were under hormonal regulation and, therefore, varied across the physiological phases of the menstrual cycle. Particularly, miRNAs targeting several cell cycle regulators were over-expressed in the midsecretory phase. Conversely, others have identified no effect on menstrual cycle phase on endometrial miRNA expression[11,53]. These discrepancies could be explained by the cell-type specificity in the response to sex steroid hormones of the human endometrium[72] and the different type of cellular populations studied in each study.

Filigheddu *et al*[61] described a set of miRNAs dif-

ferentially expressed in ovarian endometriomas in comparison to paired eutopic endometrium. By means of microarray technology, 84 significant differently expressed miRNAs were identified. In addition, the use of bioinformatic tools allowed researchers to identify the predicted targets of these dysregulated miRNAs, as well as the molecular networks and the biological function they affected. Interestingly, one of the most significantly up-regulated miRNAs was found to be miR-202-3p. In a recent report[53], our research group corroborated these results, with a 200-fold over-expression of miR-202-3p in ovarian endometriomas in comparison to paired eutopic endometrium. With regards to miR-202-3p, it has been reported[73] that this miRNA targets the glioma-associated oncogene homolog 1 (GLI1) transcription factor, a strong positive activator of downstream target genes involved in proliferation, migration, invasion and angiogenesis, such as BCL-2, CD24, metalloproteinase-2 (MMP-2) and MMP-9[74]. GLI1 also regulates the transcription of vascular endothelial growth factor A (VEGF-A), which has been postulated as the main regulator of angiogenesis[74-77]. Thus, the over-expression of BCL-2 in the eutopic endometrium of patients with endometriosis[4,67] could be a consequence of the GLI1 regulation by miR-202-3p.

Using a Next Generation Sequencing approach, Hawkins *et al*[78] found 10 miRNAs up-regulated (miR-100, -193a-3p, -193a-5p, -202, -29c, -485-3p, -509-3-5p, -574-3p, -708) and 12 miRNAs down-regulated (miR-10a, -34c-5p, -141, -200b, -200c, -200a, -203, -375, -429, -449b, -504, -873) in ovarian endometriomas in comparison to control endometrium, suggesting that miRNAs could also play a significant role in these ovarian lesions. Interestingly, one of the most dysregulated miRNAs in ovarian endometrioma was miR-29c, in agreement with our own data[53].

Different miRNA profiles have been described in peritoneal lesions compared to paired eutopic endometrial tissues[11]. Through miRNA microarray analyses and *in silico* studies, the authors identified 22 differently expressed miRNAs that putatively regulated the expression of 673 differently expressed mRNA targets. Of them, 14 were up-regulated in peritoneal lesions (miR-1, -29c, -99a, -99b, -100, -125b, -125a, -126, -143, -145, -150, -194, -223, -365) and 8 were down-regulated (miR-20a, -34c, -142-3p, -141, -196b, -200a, -200b, -424) compared to paired eutopic endometrial tissue. Interestingly, the mRNAs targets of these miRNAs had been previously related to endometriosis-associated molecular pathways, including cell death, cell proliferation and angiogenesis[11].

More recently, Saare *et al*[79] identified five over-expressed miRNAs (miR-34c, -449a, -200a, -200b, -141) in peritoneal endometriotic lesions in comparison to eutopic endometria using a high-throughput miRNA sequencing approach. This set of miRNAs allowed the discrimination of peritoneal lesions from the healthy surrounding tissue. Finally, they concluded providing a note for caution when evaluating peritoneal lesions, due that analyses carried out in biopsies also containing healthy surrounding tissues could mask aberrant miRNA expression intrinsic of peritoneal endometriotic tissues.

Although efforts have been made in the identification of the role of miRNAs in the pathogenesis of endometriosis, we are aware that future research will provide new regulatory functions for known miRNAs and that new identified miRNAs will expand our knowledge of this condition. Hence, several authors are focusing on the discovery of new miRNAs associated with human female reproductive tract disorders. For instance, Creighton *et al*[80] performed a next generation sequencing of over 100 tissues or cell lines derived from human female reproductive organs in both healthy and pathological states. As a result, 7 confirmed and 51 highly confident predicted novel miRNAs were identified.

Even though the involvement of miRNAs in the pathophysiology of endometriosis requires further investigation, nowadays these small non-coding molecules are considered as putative biomarkers for an early non-invasive diagnosis of the disease, which could suppose a valuable benefit to the patients in terms of improvement of prognosis and reduction of the ratio of recurrence, as recently demonstrated in other miRNA regulated diseases[81-83].

ANGIOGENESIS-RELATED MIRNAS IN ENDOMETRIOSIS

The involvement of angiogenesis in the physiopathology of endometrisis has been long discussed, as the endometrial tissue migrated to the peritoneum requires a blood supply in order to survive, proliferate, invade the extracellular matrix and establish the endometriotic lesion[84]. VEGF represents one of the most potent angiogenic factors. Several studies have reported an increase in VEGF-A levels in endometriosis and it has been suggested that VEGF-A plays an important role in the progression of the disease[84,85]. Regarding angiogenesis inhibitors, alterations of thrombospondin-1 (TSP-1) expression has been reported to be involved in endometriosis, in which vascularisation is mandatory for the survival of migrated tissues[85].

In previous publications, our research group has found and up-regulation of the expression of angiogenic and proteolytic factors in endometrial tissue from patients with endometriosis[85,86] and we have suggested that this increase might contribute to the invasive potential of endometrial cells.

The miRNA regulation of angiogenesis has been long reported in several pathologies, including endometriosis[12,53,54]. The miR-17-92 cluster, also known as oncomir-1, encodes six mature miRNAs (miR-17, miR-18a, miR-19a, miR-19b, miR-20a and miR-92a)[87] and has been reported to play an important role in the tumor neovascularisation[27]. Two miRNAs encoded in this cluster, miR-17-5p and miR-20a, have been found

to be down-regulated in ovarian endometriotic cysts in comparison to eutopic endometrium[11,61]. As miR-17-5p targets TSP-1, a decrease of the miR-17-5p levels in ovarian cysts could repress the down-regulation of TSP-1 expression and provide an explanation for the clinically observed low invasion grade of these endometriotic lesions to the surrounding ovarian tissue.

Extracellular matrix remodelling is a crucial process in the regulation of angiogenesis[88] and plays a critically important role in the establishment of the endometriotic lesion[89]. mRNA levels of key components of the metalloproteinase systems have been reportedly observed to be deregulated in eutopic and ectopic endometria of patients with endometiosis[85,86,89,90]. In a recently published paper[53], we observed that miR-29c-3p was up-regulated in several endometriosis tissues (ovarian endometrioma, peritoneal lesions and rectovaginal nodule). Provided that miR-29c-3p regulates different genes of the extracellular matrix, our results are in agreement with previously published studies[11,78] accounting for the coordinated role of several miRNAs in the remodelling process necessary for the implantation of migrated endometria in ectopic locations and the establishment of endometriotic lesions.

Taking into consideration the importance of angiogenesis in the pathophisiology of endometriosis, several therapies targeting VEGF as blockers or inhibitors have been proposed aiming to decrease the number of lesions, inhibit growth and reduce vascular density. In this context, soluble truncated VEGF receptors (Flt-1), antibodies to human VEGF and bevacizumab[91], among others, have been tested in murine models of endometriosis. Although results from these studies are promising, it should be taken into account that the use of an animal model that neither menstruate nor develop spontaneous endometriosis is a major limitation. Furthermore, the use of molecules that might block the expression or mimic functions of angiogenesis-related miRNAs could represent new therapeutic approaches in the treatment of endometriosis as recently demonstrated in other miRNA-regulated diseases[92].

CLINICAL UTILITY OF MIRNAS AS BIOMARKERS OF ENDOMETRIOSIS

Despite the fact that endometriosis is one of the most common benign gynecological diseases, there is a lack of non-invasive or semi-invasive diagnostic test that overcomes the need for the current surgical diagnosis[53,67]. Laparoscopy with histological confirmation, the gold standard for diagnosis, is a minimally invasive procedure. However, patients usually undergo general anesthesia and a certain degree of expertise from clinicians is necessary and it is a costly procedure. Additional concerns are related to the delay in the diagnosis of the disease, which has been estimated of around 7 years[32,93]. This may be due to multiple reasons including non-specific symptoms of the disease (pelvic pain and infertility), which leads to multiple tests for differential diagnosis[45]. As a consequence, patients are diagnosed at advanced stages of the disease, which impairs the prognosis and increases the risk of recurrence. For all these reasons, there is a great interest among researchers to find a non-invasive or semi-invasive test for the diagnosis of endometriosis that would ideally diagnose patients in initial steps of the disease and overcome the need for an invasive procedure.

Since they were first described to be present in blood[82], circulating miRNAs have become interesting biomarkers in different conditions[16,23,24,94,95]. The presence of miRNAs in different biofluids, including blood[96], could be explained by different mechanisms: (1) passive release of miRNAs from broken cells and tissues following tissue injury, chronic inflammation, cell apoptosis or necrosis, or from cells with a short half-life, such as platelets; (2) active secretion *via* cell-derived microvesicles (including exosomes and shedding vesicles); and (3) active secretion by cells as RNA-binding-protein conjugated complexes. Mechanisms (2) and (3) also offer an explanation for their highly elevated stability in plasma, despite the presence of elevated amounts of RNAses[97]. Although so far the biological functions of circulating miRNAs remain to be completely defined, some authors have proposed a role into cell-to-cell communication for these short nucleic acids[97-100]. In any case, it is clear that their presence in plasma/serum and the distinct advantages that they offer over other biomarkers (for instance and unlike mRNAs, miRNAs show high stability in blood, can be both amplified and detected with high sensitivity and specificity[101] and are highly resistant to storage handling[97,101]) offers an opportunity to use them as biomarkers.

In the field of gynecological pathologies, several authors have explored this possibility[94,102,103]. In ovarian cancer, miRNA expression profiles have been analyzed in whole blood and sera from patients, either as free-circulating miRNAs or encapsulated in exosomes. An example of the last is the study conducted by Taylor *et al*[95] in serum exosomes from patients with serous papillary adenocarcinoma of the ovary. Eight miRNAs (miR-21, -141, -200a, -200b, -200c, -203, -205, -214) were found to be up-regulated in tumor-derived exosomes compared with serum from benign ovarian disease patients. Interestingly, these 8 miRNAs showed a high correlation between their cellular and exosomal levels. In another study, published by Resnick and coworkers[104], 8 miRNAs were found to be deregulated (miR-21, miR-29a, miR-92, miR-93 and miR-126 up-regulated and miR-99b, miR-127 and miR-155 down-regulated) in serum obtained from 19 patients with epithelial ovarian carcinoma (serous, clear cell, endometrioid and mucinous) in comparison to miRNAs analysed in sera from 11 controls. Interestingly, three out of the five up-regulated miRNAs (miR-21, miR-92

Table 1 Current studies assessing the clinical utility of circulating miRNAs as biomarkers of endometriosis

Sample	Anticoagulant	Main results	Participants (patients/controls)	Ref.
Serum	-	↓ let-7b and miR-135[a] let-7d and let-7f showed a tendency towards down-regulation[a]	$n = 24/n = 24$[b]	[102]
Plasma	EDTA	↓ miR-17-5p, miR-20a and miR-22	$n = 23/n = 23$[c]	[103]
Serum	-	↑ miR-122 and miR-199a ↓ miR-9*, miR-141*, miR-145* and miR-542-3p[a]	$n = 60/n = 25$[d]	[106]
Plasma	EDTA	↓ miR-200a-3p, miR-200b-3p and miR-141-3p[a]	$n = 61/n = 65$[e]	[110]

Down-regulated (↓) and (↑) up-regulated miRNAs in samples from patients in comparison to control women. [a]Combination of miRNAs in bold yielded the best diagnostic value; [b]Control group presented dermoid cysts ($n = 10$), serours cystadenoma ($n = 5$), mucinous cystadenoma ($n = 3$), simple ovarian cysts ($n = 3$) and paratubal cysts ($n = 1$); [c]Control group presented uterine leiomyoma ($n = 14$), mature teratoma ($n = 4$), simple cysts ($n = 3$) and unexplained infertility ($n = 2$); [d]Main diagnosis: Infertility due to tubal factors; [e]Thirty-five endometriosis-free women with primary ($n = 10$) or secondary ($n = 15$) infertility, suspicion of endometriosis ($n = 5$), polycystic ovaries ($n = 3$) and pelvic pain ($n = 2$) and 30 self-reported healthy women. EDTA: Ethylenediaminetetraacetic acid.

and miR-93) were overexpressed in 3 patients with normal CA-125 levels. This finding could be explained by the high sensitivity and accurancy of the RT-PCR quantification, suggesting that miRNAs could provide an advantage as biomarkers in terms of sensitivity in comparison to those in current clinical use.

Häusler *et al*[105] analysed the miRNA expression profile in whole blood from 24 patients with epithelial carcinoma (mainly serous histotype) and from 15 healthy donors. As a result, the expression of miR-30c1* was found to be up-regulated and the expression of miR-181a*, miR-342-3p and miR-450b-5p down-regulated in patients in comparison to controls, enabling a discrimination between populations.

Regarding endometriosis, an interesting recent review from Fassbender *et al*[23] pointed to the possibility of developing a semi-invasive test for endometriosis from PF obtained *via* transvaginal ultrasound-guided aspiration. Although this is an interesting approach, current research is mainly focused on developing serum/plasma biomarkers as a noninvasive diagnotic tool. Jia *et al*[103] explored this possibility, conduct a study that enrolled 23 women with histologically proven endometriosis and 23 endometriosis-free controls. RNA from plasma was extracted to perform a miRNA microarray profiling. Three out of the six miRNAs selected for qRT-PCR (miR-17-5p, miR-20a and miR-22) were proven to be significantly down-regulated in patients and useful to discriminate women with endometriosis from patients. Wang *et al*[106] performed a circulating miRNA profiling with a different approach. For miRNA profiling, 2 pools of sera from 10 endometriosis patients and 10 control women, respectively, were prepared. Results from array were validated by qRT-PCR in sera from 60 patients and 25 control women, finding that miR-199a and miR-122 levels were up-regulated and miR-145*, miR-141*, miR-542-3p and miR-9* down-regulated in samples from patients in comparison to control women and could therefore serve as biomarkers of the disease. In a very recent study, Cho *et al*[102] quantified the levels of miR-135a,b and let-7a-f in sera of 24 endometriosis patients and 24 disease-free women. The selection of these miRNAs was based on their previous association with endometriosis[107,108]. Employing a logistic regression approach, researchers found that a combination of let-7b, let-7d and let-7f during the proliferative phase yielded the highest area under the curve value in discriminating patients with endometriosis from control women. Of note, several miRNAs were found to be differently expressed depending on the phase of the menstrual cycle in patients but not in controls, in agreement with previous reports[109]. Finally, Rekker *et al*[110] performed the last published study regarding circulating miRNAs as biomarkers of endometriosis. Based on previous literature, authors selected 3 miRNAs from the miR-200 family (miR-200a-3p, miR-200b-3p and miR-141-3p) whose expression was assessed in plasma samples from 61 patients and 65 control women. The expression of all 3 miRNAs was down-regulated in patients and miR-200a-3p and miR-141-3p showed the highest potential as noninvasive biomarkers for this benign condition. Remarkably, authors also analyzed variations of the levels of the three miRNAs of interest with time of sampling (morning/evening) finding lower levels in evening samples, perhaps due to circadian fluctuations in their expression. This is an interesting approach and points to the time of sampling as an important factor to be taken into account when performing circulating miRNAs studies. All these studies on the role of circulating miRNAs as biomarkers of endometriosis are summarized in Table 1.

Importantly, it should be noted that the circulating miRNA pool is not a mirror of tissue miRNAs content[83,111] and that changes in tissue miRNA will not be reflected in the same extent in the circulating miRNA profile[101]. Therefore, the aforementioned differences in endometrial miRNA expression profiles found in endometriosis should be considered in the context of a semi-invasive diagnosis of endometriosis by means of endometrial biopsy, because of the low probability of finding such differences in serum or plasma from the same patients.

CONCLUSION

MiRNAs, one of the epigenetic players, are small non-coding RNAs that can act as post-transcriptional regulators of gene expression reducing the expression of their target mRNA. The involvement of miRNAs in

different pathological conditions has been well established and miRNA expression profiles have been performed in biopsies from different conditions, including gynaecological pathologies as endometriosis. Despite being a benign gynaecological pathology, endometriosis deeply impairs the quality of life of affected women in terms of pain and infertility. The prevalence of endometriosis in reproductive-age women is estimated around 1 out of 10 and raises to 5 out of 10 in patients experiencing both pain and infertility. Research endeavours are being conducted in order to find a non-invasive or semi-invasive biomarker of the disease that ideally diagnosis the disease at initial stages and overcomes the need for the current laparoscopy gold standard diagnosis. In this area, circulating miRNAs have emerged as attractive molecules to be considered as biomarkers. Up to date, only few studies have been performed in order to obtain a circulating miRNA-based diagnostic tool. However, differences in experimental design among them make it difficult to compare results. From our point of view, there is a need for standardization of clinical data annotation, sample collection and handling among research projects that takes into account several aspects: (1) surgical and non-surgical data; (2) type of sample (serum/plasma) and processing protocols. In the case of plasma, the choice of anticoagulant is not a minor feature in experimental design and must be carefully addressed; (3) time of sampling is also an important factor and a decision has to be made between morning fasting samples or evening samples, as demonstrated by Rekker *et al*[110]; and (4) number of participants in circulating miRNAs as biomarkers of endometriosis studies is scarce and usually control population is heterogeneous, including self-reported endometriosis-free women, patients with different benign gynaecological conditions and infertile women due to tubal factors. For all these reasons, we encourage researchers in the field to follow recommendations from the World Endometriosis Research Foundation[112-115] in order to solve the observed heterogeneity in experimental designs and improve reproducibility between studies. In addition, validation of experimental algorithms in different cohorts is needed so as to improve quality of research and reach the ultimate goal, benefit patients with an earlier diagnose of endometriosis and avoiding unnecessary assisted reproductive techniques in those women whose fertility is not affected by the disease. To achieve this ambitious objective, we do encourage researchers to collaborate and synergistically add efforts to be able to recruit larger cohorts of patients and endometriosis-free women for circulating miRNAs studies, adopt standardized protocols and improve research outcomes.

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microRNAs and angiogenesis in endometriosis

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ABSTRACT

miRNAs function as important regulators of a wide range of cellular processes, such as angiogenesis and fibrinolysis, by postranscriptional modulation of gene expression. We present a review on the role of miRNAs and angiogenesis in endometriosis.

Endometriosis, defined as the implantation of endometrial tissue outside the uterine cavity, is one of the most frequent benign gynecological diseases and it has important consequences on the quality of life and fertility of patients. Similarly to tumor metastasis, the ectopic endometrium acquires the capability to adhere, proliferate and infiltrate the extracellular matrix. Endometriosis is a multifactorial and polygenic disease in which angiogenesis and proteolysis may be involved, and emerging data provide evidence that a dysregulation of miRNA expression may be implicated in these processes. The detection of circulating miRNAs in plasma and other body fluids and their relative stability has raised the possibility that they might serve as non-invasive biomarkers for the diagnosis of the disease. On the other hand, the development of therapies that might block the expression or mimic the functions of miRNAs could represent new therapeutic strategies for the treatment of endometriosis.

microRNAs

microRNAs (miRNAs) are small 21–22 nucleotide non-coding RNAs that regulate gene expression and play a key role in a wide range of biological processes. These small molecules bind to the 3' untranslated regions (3'-UTR) of their target mRNAs, mediating translational repression and/or mRNA degradation [1,2].

Although miRNAs were first discovered in 1993, it has been more recently when research endeavors have suggested and reinforced their role as important regulators of gene expression in most cellular processes and a broad spectrum of diseases [3,4].

Several conditions of miRNAs provide them the capability to act as ideal biomarkers to assess the presence or prognosis of several gynecological diseases. miRNAs lack of known post-processing modifications, have low complexity and are present at diverse body fluids [5]. miRNAs can be analyzed not only in biological fluids (plasma, serum, peritoneal fluid, etc) but also in fresh frozen tissues and in formalin-fixed paraffin-embedded tissues due to their stability in adverse conditions.

Abnormal miRNA expression profiles are associated with several human diseases, including cancer, cardiovascular disorders and benign or malignant disorders of the human female reproductive tract [4,6–8]. The altered miRNA expression pattern in women with endometriosis may promote ovarian cancer development by enhancing or inactivating different oncogenic and tumor suppressor target genes [9]. Thus, this miRNA dysregulation could give the rationale for the observed two-fold increased risk for ovarian cancer reported in this population [10,11]. Therefore, unraveling the role of miRNAs in gynecological diseases, such as ovarian and endometrial cancer, might provide important tools to ascertain the potential of malignant transformation in endometriosis.

microRNAs and angiogenesis in endometriosis

Endometriosis is defined by the presence of endometrial glands and stroma outside the uterine cavity affecting up to half of the patients with pain and infertility and resulting in important impairment of their quality of life. To establish the endometriotic lesion, the ectopic endometrium has to survive outside the uterus, avoid the immunity mechanisms and, mimicking tumor metastases, attach to the peritoneum or other locations, infiltrate the extracellular matrix and create a vessel network through activation of angiogenesis [12]. Endometriosis includes a wide range of lesions at different locations and three different entities can be differentiated: endometriotic implants on the surface of the peritoneum (peritoneal endometriosis), ovarian cysts lined by endometrioid glands (ovarian endometriomas), and fibrotic masses comprising endometriotic, adipose and fibromuscular tissues, usually located

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between the rectum and the vagina (rectovaginal endometriotic nodule). Moreover, peritoneal implants can be divided in active or red lesions (highly vascularized) and inactive or black lesions (more fibrotic). Although it remains uncertain whether these three types are variants of the same pathologic process or caused by different mechanisms [13], anyhow it seems clear, that their specific clinical features warrant an individualized analysis of their biological behavior.

Endometriosis is a benign disease that behaves in several aspects such as cancer. For instance, endometriotic tissue metastasizes outside the uterine cavity and infiltrates the surrounding tissue progressing along the time if no therapy is adopted. This disease is classified as a tumor-like entity by the World Health Organization Histologic Classification of Ovarian Tumors [14]. In 1925, Sampson was the first to report a case of a suspected malignant transformation in endometriosis [15]. Since then, several studies have focused on the relationship between endometriosis and gynecological cancers, especially ovarian cancer [16].

Endometriosis has been described as a multifactorial and polygenic disease and emerging data provide evidence that dysregulation of miRNA expression may be involved [17–23]. miRNAs appear to be potent regulators of gene expression in endometriosis, raising the prospect of using miRNAs as biomarkers and therapeutic tools of the disease [24].

It has been reported that angiogenesis may play an important role in the pathogenesis of endometriosis. Similarly to tumor metastases, endometriotic implants require neovascularization to proliferate, invade the extracellular matrix and establish an endometriotic lesion [12]. Vascular endothelial growth factor (VEGF) represents one of the most potent angiogenic factors. Several studies have reported an increase in VEGF-A levels in endometriosis and it has been suggested that VEGF plays an important role in the progression of the disease [8,19]. Thrombospondin-1 (TSP-1) is an inhibitor of angiogenesis and it has been reported that alterations in TSP-1 expression may be involved in many pathologies of the reproductive tract, including endometriosis, in which vessel formation occurs [8].

We have previously reported an increase in the expression of angiogenic and proteolytic factors in endometrial tissue from patients with endometriosis [17–20] and we have suggested that this increase might contribute to the invasive potential of endometrial cells.

The miRNA expression profiles of eutopic and ectopic endometrium (peritoneal and ovarian endometrioma) from women with endometriosis have been recently described [24]. Two independent studies reported that miR-17-5p and miR-20a, involved in angiogenesis regulation, are down-regulated in the ovarian endometrioma in comparison to eutopic endometrium [25,26].The miR-17-92 cluster, also known as oncomir-1, encodes six mature miRNAs (miR-17, miR-18a, miR-19a, miR-19b, miR-20a and miR-92a) [27]. It has been reported that the miR-17-92 cluster increases tumor neovascularization by down-regulating TSP-1 expression [28]. Therefore, a decrease in miR-17-5p levels could in turn post-transcriptionally up-regulate TSP-1 expression. In keeping with some previously mentioned authors, this mechanism may reduce the angiogenic activity of the ectopic endometrium in the ovarian endometrioma, thus helping to explain the low invasiveness of this tissue and also the frequent clinical finding of a preserved ovarian tissue surrounding the ovarian endometriotic cyst.

Our group has confirmed these findings in recent studies [8,29] in which the miRNA expression profiles of eutopic endometrium from women without endometriosis as well as of paired eutopic and ovarian endometrioma from patients were obtained. In addition, we have showed a significant increase in miR-125a, miR-222 and miR-202-3p levels and a decrease in VEGF-A protein levels in ovarian endometrioma in comparison to eutopic endometrium. Furthermore, miR-202-3p expression was significantly lower in eutopic endometrium samples. It has been reported [30] that miR-202-3p down-regulates the expression of the proteinglioma-associated oncogene homolog 1 (GLI1), a transcription factor that, in turn, regulates the transcription of both Bcl-2 and VEGF-A [30–33]. Accordingly, dysregulation of miR-202-3p could be, at least in part, responsible for the increased VEGF-A levels in eutopic endometrium from patients and reduced VEGF-A levels observed in ovarian endometrioma. On the other hand, the overexpression of Bcl-2 found in the eutopic endometrium of women with endometriosis [34,35] could be modulated by miR-202-3p by regulating the expression of the GLI1 transcription factor. These findings suggest that dysregulation of miRNA during endometriosis might play a pivotal role establishing endometriotic lesions by affecting different physiological processes.

Endometriosis is a multifactorial disease in which endometrial and peritoneal factors could be involved. Peritoneal fluid from patients with endometriosis is a complex suspension carrying inflammatory cytokines, growth factors, steroid hormones, proangiogenic factors, macrophages, and endometrial and red blood cells. Several years ago, our group described increased VEGF-A levels in this body fluid from patients [19,23], which could be correlated with the stage of the disease. Moreover, results from our group indicate that the peritoneal fluid from patients reduces the expression of VEGFA-related miRNAs miR-16, miR-17-5p, miR-20a and miR-125a in primary stromal cell cultures from affected women. Together with the parallel increase observed in VEGF-A these findings suggest that peritoneal fluid from patients influences endometriotic cell survival through the dysregulation of angiogenesis-related miRNAs [28]. Therefore, the angiogenic potential of both the endometrium and the peritoneal environment found in endometriosis patients could promote lesion establishment and survival.

Extracellular matrix proteins remodelling also plays a critically important role in the establishment of the endometriotic lesion. An abnormal expression of different components of the metalloproteinase systems both in the endometrium and in the endometriotic tissue of women affected with the disease has been reported [20]. Thus, miR-29c is up-regulated in the endometriotic tissue and its role targeting different extracellular matrix genes supports a potential role of the miRNAs network in the remodelling process that leads to implantation of endometrial tissue outside the uterus and to the formation of early endometriotic lesions [26].

Several therapies targeting VEGF as blockers or inhibitors have been proposed aiming to decrease the number of lesions, inhibit growth and reduce vascular density. In this context, soluble truncated VEGF receptors (Flt-1), antibodies to human VEGF and bevacizumab [36], among others, have been tested in murine models of endometriosis. Although results from these studies are promising, it should be taken into account that the use of an animal model that neither menstruate nor develop spontaneous endometriosis is a major limitation. Furthermore, the use of molecules that might block the expression or mimic functions of angiogenesis-related miRNAs could represent new approaches to guide therapeutic interventions in endometriosis, as recently demonstrated in other miRNA-regulated diseases [28].

Future perspectives

Despite its high prevalence, endometriosis is associated with a delayed diagnosis of several years, perhaps because the gold standard for diagnosis is surgical assessment by laparoscopy or laparotomy. miRNAs have been proposed as biomarkers for several diseases including endometriosis, endometrial and ovarian cancer. In this context, the evaluation of miRNAs in minimally invasive samples as menstrual blood or plasma could represent a useful tool for an early diagnosis and an improved management of

this condition. In addition, miRNAs could be a useful tool for the treatment of these diseases or they could be implicated in pharmacological treatment responses. Moreover, the development of therapies that modulate miRNA expression, either blocking or mimicking the miRNA activity could represent new therapeutic strategies for any of the aforementioned gynecological disorders.

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Conflict of interest statement

The authors have no conflicts of interest to declare.

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2. Papers not directly related to the Doctoral Thesis:

1. Braza-Boïls A, Marí-Alexandre J, Molina P, Arnau MA, Barceló-Molina M, Domingo D, Girbes J, Giner J, Martínez-DOlz L, Zorio E. Deregulated hepatic microRNAs underlie the association between non-alcoholic fatty liver disease and coronary artery disease. Liver Int 2016; 36:1221-1229

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ORIGINAL ARTICLE

Deregulated hepatic microRNAs underlie the association between non-alcoholic fatty liver disease and coronary artery disease

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Abstract

Background & Aims: Non-alcoholic fatty liver disease (NAFLD) appears to be a new risk factor for the development of coronary artery disease (CAD). Members of a class of non-coding RNAs, termed microRNAs (miRNAs), have been identified as post-transcriptional regulators of cholesterol homoeostasis and can contribute to the development of NAFLD. The aims of this study were to (i) to assess the relationship between NAFLD and sudden cardiac death (SCD) from severe CAD in forensic autopsies and (ii) to quantify several hepatic miRNAs previously associated with lipid metabolism and NAFLD to correlate their expression with the presence of NAFLD, CAD, obesity parameters and *postmortem* lipid profile. *Methods:* A total of 133 cases of autopsies with SCD and established CAD (patient group, CAD-SCD) and 106 cases of non-CAD sudden death (control group, non-CAD-SD) were included. miRNAs were quantified in frozen liver tissues. *Results:* Males predominated in both groups. Patients more frequently exhibited NAFLD and necroinflammatory steatohepatitis (NASH) than controls (62% vs 26%, $P = 0.001$ and 42% vs 26%, $P = 0.001$ respectively). In both groups, the presence of NAFLD correlated with body mass index and abdominal circumference ($P < 0.05$). An increase in miR-34a-5p and a decrease in miR-122-5p and -29c-3p in patients with NASH vs controls without NAFLD were observed ($P < 0.05$). Finally, significant correlations between miR-122-5p and unfavourable lipid profile and also hs-CRP and miR-34a-5p were noted. *Conclusions:* CAD is associated with NAFLD and NASH. The hepatic miRNAs studied appear to be associated with NAFLD severity and may promote CAD through lipid metabolism alteration and/or promotion of the systemic inflammation.

Keywords
ischaemic heart disease – microRNAs – steatohepatitis – sudden cardiac death

Sudden cardiac death (SCD) is the leading cause of sudden unexpected non-traumatic deaths in adults aged <65 years in Western countries, being the first manifestation of cardiovascular disease in 20–40% of the cases. Although the incidence of SCD varies in different series regarding a variable range of ages, it has been estimated in 0.35–1.28 per 1000 inhabitants per year (1) and it has become a major public health problem in industrialized countries. Coronary artery disease (CAD) is the most prevalent cause of SCD, being responsible for 60–80% of the cases of SCD (2).

Abbreviations
AC, abdominal circumference; BMI, body mass index; CAD, coronary artery disease; GGT, ɣ-glutamyl transpeptidase; miRNA, microRNA; NAFLD, non-alcoholic fatty liver disease; NASH, necroinflammatory steatohepatitis; SCD, sudden cardiac death.

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Key points

- The presence and the severity of non-alcoholic fatty liver disease (NAFLD) are associated to the presence of significant coronary stenoses in victims of sudden cardiac death.
- A deregulation of several hepatic microRNAs (namely miR-34a-5p, -122-5p and -29c-3p) has been observed in association not only to the development of NAFLD but also to its severity.
- The miR-34a-5p is upregulated also in healthy livers of patients with coronary stenoses.
- As a speculative connection, our results suggest that an unfavourable lipid profile and a proinflammatory effect could underlie the essentials of the cross-talk between the liver (with NAFLD) and the coronary arteries (with significant stenoses).

Non-alcoholic fatty liver disease (NAFLD) is a clinical and pathological multifactorial disorder characterized by similar histological findings to those observed in alcoholic hepatitis but in the absence of alcohol intake in amounts known to cause hepatic damage (less than 20 g/day is commonly accepted) (3). NAFLD ranges from simple steatosis to necroinflammatory steatohepatitis (NASH) with different degrees of severity and fibrosis. Although NAFLD represents the most common cause of liver disease in the Western world being its prevalence 13–30% in the general population (4, 5), the estimated prevalence of NASH is only 2–3% in the general population (6) and 37% in the morbidly obese patients (7).

The association between NAFLD and obesity, type 2 diabetes mellitus and/or dyslipidaemias is being increasingly recognized supporting the new concept that NAFLD could be regarded as a true new component of the metabolic syndrome or even an active factor involved in its pathogenesis (8–10). The increased cardiovascular risk observed in NAFLD patients has suggested a predisposition to atherosclerosis although it is still a matter of debate whether this link is dependent or independent from other features of the metabolic syndrome (10). The possible mechanisms linking NAFLD and CAD include inflammation and oxidative stress pathways, hyperlipidaemia and insulin resistance (8–11).

Besides the classical factors involved in cholesterol metabolism, microRNAs (miRNAs) have been identified as important post-transcriptional regulators of cholesterol homoeostasis (12). miRNAs are small (21–22 nucleotides) non-coding RNAs that regulate gene expression and play fundamental regulatory roles in many pathophysiological processes (13). These small molecules bind to target mRNAs, mediating translational repression and/or mRNA degradation (13–15). Several studies have identified a specific miRNA expression profile associated to NAFLD (16–18) and others have suggested the diagnostic potential of miRNAs in CAD (19, 20).

Pathological information derived from *postmortem* studies constitutes a key source of knowledge to understand many disease conditions. According to the legislation, a forensic autopsy is required in all clear or suspected violent deaths, including sudden and unexpected deaths in non-hospitalized children and young adults. Forensic-based studies could provide relevant data to develop cardiovascular prevention strategies. Only few reports on autopsy series have corroborated that ischaemic heart disease is the most common cause of death among autopsies of patients with NAFLD (21, 22) and none has simultaneously analysed the potential role of miRNAs in this scenario. The aim of this study has been not only to assess the relationship between NAFLD and severe CAD in forensic autopsies in a county of Spain, but also to correlate the level of certain hepatic miRNAs with the presence of NAFLD, CAD, obesity and dyslipidaemia.

Material and methods

Ethics statement

This study was approved by the Ethical Committee from the Hospital Universitario y Politécnico La Fe, Valencia, Spain (#2013/0113).

Clinical Groups

A total of 133 consecutive cases of SCD associated with significant CAD (CAD-SCD, patient group) and 106 consecutive cases of non-CAD unexpected sudden deaths (non-CAD-SD, control group) were prospectively included from 2008 to 2014 following a broad protocol created to offer a multidisciplinary approach of SCD occurring under 55-year old. Autopsies were performed at the Institute of Legal Medicine of Valencia, Spain. Sudden death was defined as a natural death either (i) witnessed and occurring within 1 hour from the onset of symptoms in an apparently healthy subject or whose disease was not so severe as to predict such an abrupt outcome or (ii) unwitnessed when the deceased was known to be in good health 24 h before death (1, 23). CAD-SCD (patient group) was defined by at least one of the following: an atheromatous cross-sectional area reduction of >75% in at least one epicardial coronary artery, a complicated plaque (ruptured or eroded with or without thrombosis) and the presence of acute or healed myocardial infarction. Non-CAD-SD (control group) comprised 43 cases of SCD with structurally normal heart, 21 familial cardiomyopathies (hypertrophic, arrhythmogenic and dilated cardiomyopathies), 6 pulmonary thromboembolisms, 5 aortic dissections, 4 valvulopathies, 3 myocarditis, 2

channelopathies, 1 coronary dissection and 1 Wolf–Parkinson–White syndrome and 20 non cardiac causes including brain haemorrhages and thoracic traumatisms. Forensic investigation included compilation of circumstances of death and premortem clinical information, routine autopsy with body mass index (BMI), abdominal circumference (AC), routine toxicological analyses and macro and microscopic examination of the major organs. *Brunt* histological scoring system semiquantively assessed the presence of steatosis, inflammation and hepatocytic injury to grade NASH severity (grade 1 mild, grade 2 moderate and grade 3 severe) (24). The cause of death was established based on the correlation of clinical and pathological reports following current recommendation and guidelines (1). BMI was calculated from weight in kilograms divided by the square of height in meters. This study protocol conforms to the ethical guidelines of the 1975 Declaration of Helsinki and was approved by our institution's human research committee. No written informed consent was required given the fact that all the participants were dead at the time of the recruitment and that all the biological samples analysed had been obtained within the protocol of the legal autopsy that they all underwent following the Spanish Laws concerning sudden deaths.

Analytical parameters

Analytical parameters were analysed in peripheral blood whenever it was available at the postmortem examination. Total cholesterol (T-Ch), triglycerides and GGT were determined by enzymatic methods and HDL-cholesterol (HDL-Ch) with direct methods (Architect 16000; Abbott Diagnostic, Lake Forest, IL, USA). When triglycerides were <400 mg/dl LDL-cholesterol (LDL-Ch) was calculated with Friedewald's formula. VLDL-cholesterol (VLDL-Ch) was obtained from triglycerides/5. Lipoprotein(a) and apolipoprotein A and B and were all measured using kinetic nephelometry (Immage Nephelometer®; Beckman Coulter Inc., Brea, CA, USA). High-sensitivity C reactive protein level (hs-CRP) was assessed with turbidimetric methods (Immage Nephelometer®; Beckman Coulter Inc.).

RNA extraction and quality determination

Liver tissues were rinsed in cold PBS and stored in RNA later (Ambion) at −80°C until their processing. Liver samples from 68 patients and 27 controls were included in the miRNA study. The mirVana-Paris miRNA isolation kit (Ambion, Austin, TX, USA) was used to extract the total RNA. The RNA concentration and purity were determined using a NanoDrop ND-1000 spectrophotometer (Nanodrop Technologies, Wilmington, DE) in all the samples. Following manufacturer's specifications, only samples with A260/A280 ratio of ~2.0 and A260/230 ratio in the range 1.9–2.2 were considered for inclusion in this study.

Quantification of selected mature miRNAs by quantitative real-time RT-PCR

On the basis of the literature supporting the association of miRNAs with NAFLD, we selected six miRNAs to be evaluated in our cohort of samples: miR-122-5p (MIMAT0000421), -33a-5p (MIMAT0000091), -34a-5p (MIMAT0000255), -21-5p (MIMAT0000076), -29c-3p (MIMAT0000681) and -106b-5p (MIMAT0000680). The miR-27a-3p (MIMAT0000084), but not the small nucleolar RNA RNU6B, served as an endogenous control since RNU6B did not present stable levels in our samples. Instead, the miR-27a-3p was tested and validated as an endogenous control.

Quantification was performed by miRCURY LNA™ Universal RT microRNA PCR (Exiqon, Vedbaek, Denmark). This method is based on a universal RT followed by a real-time PCR amplification with LNA™ enhanced primers. The protocol was performed as outlined in the instruction manual, employing a LightCycler 480 instrument II (Roche, Applied Science, Penzberg, Germany).

Statistical analysis

Adequate variable measures of central tendency and dispersion were used and chi-square, odds ratio, Student's *t*-test and ANOVA (with Bonferroni correction) were calculated to assess associations of dichotomic and continuous variables. Pearson's test was used for correlation assessments. In the analyses, SPSS 12.0 software (SPSS, Inc., Chicago, IL, USA) was used. Multivariate analyses were performed including in the model the epidemiological variables with statistically significant differences in the univariate, namely age, sex, BMI and AC.

miRNA data quantified by real-time qRT-PCR are presented as fold changes with respect to the reference subgroup (the controls without NAFLD). Values are expressed as mean ± standard error of the mean (SEM).

Results

Clinical and forensic data from our 239 cases are shown in Table 1. Patients were significantly older than controls (45 ± 8 vs 37 ± 10, $P < 0.001$). In both groups, a male predominance was observed (88% in patients and 73% in controls). BMI (27.1 ± 4.3 kg/m^2 vs 25.7 ± 4.7 kg/m^2, $P < 0.05$) and AC (95.2 ± 13.1 cm vs 90.0 ± 15.1 cm, $P < 0.01$) were again higher in the patient than in the control group. Among the analytical parameters, CAD-SCD patients exhibited increased levels of T-Ch (204 ± 62 mg/dl vs 175 ± 56 mg/dl, $P < 0.05$), T-Ch/HDL-Ch ratio (5.9 ± 2.3 vs 4.5 ± 1.3, $P < 0.01$) and LDL-Ch/HDL-Ch ratio (3.4 ± 1.4 vs 2.4 ± 1.4, $P < 0.05$) (Table 1).

Table 1. Anthropometric, lipid and other analytic parameters in patients (CAD-SCD group) vs controls (non-CAD-SD group)

Total Groups	CAD-SCD group (*N* = 133)	Non-CAD-SD group (*N* = 106)	*P*
Age (years)	45 ± 8	37 ± 10	<0.001
Male/female	117/16	77/29	0.004
BMI (kg/m^2)	27.1 ± 4.3	25.7 ± 4.7	0.027
AC (cm)	95.2 ± 13.1	90.0 ± 15.1	0.009
	(*N* = 66)	(*N* = 35)	*P*
T-Ch (mg/dl)	204 ± 62	175 ± 56	0.041
HDL-Ch (mg/dl)	37 ± 12	40 ± 14	0.273
LDL-Ch (mg/dl)	120 ± 52	90 ± 49	0.061
VLDL-Ch (mg/dl)	88 ± 42	72 ± 21	0.073
Triglycerides (mg/dl)	433 ± 214	359 ± 107	0.107
T-Ch/HDL-Ch ratio	5.9 ± 2.3	4.5 ± 1.3	0.007
LDL-Ch/HDL-Ch ratio	3.4 ± 1.4	2.4 ± 1.4	0.032
ApoA1/ApoB ratio	1.1 ± 0.31	1.3 ± 0.52	0.058
Lipoprotein (a) (mg/dl)	37.9 ± 31.1 (0–124)	41.1 ± 73.4 (2–344)	0.158
hs- CRP (mg/dl)	9.8 ± 19.0	5.6 ± 19.9	0.381
GGT (IU/l)	59 ± 35	50 ± 35	0.305

BMI, body mass index; AC, abdominal circumference; T-Ch, total cholesterol; HDL-CH, High-density lipoprotein cholesterol; LDL-Ch, low-density lipoprotein cholesterol; VLDL-Ch, very-low-density lipoprotein cholesterol; hs-CRP, high-sensitivity C reactive protein; GGT, ɣ-glutamyl transpeptidase.

Prevalence of NAFLD

The presence of NAFLD was associated with CAD in our study population (62% vs 26%, $P < 0.001$) with an odds ratio for CAD-SCD of 4.62 (95% CI 2.65–8.07) (Table 2). Furthermore, when grading the presence of NAFLD into absence, steatosis and NASH, a higher percentage of CAD-SCD individuals was observed in the two latter (and more unfavourable) groups among patients in comparison to controls with increasing odds ratios attending to the severity of the NASH in the univariate and also in the multivariate analyses (Table 2).

Expression of hepatic miRNAs measured in liver of a subgroup of patients and controls

The selected six miRNAs (miR-122-5p, -33a-5p, -34a-5p, -21-5p, -29c-3p, -106b-5p) were quantified only in 68 cases of the patient and 27 cases of the control group from whom frozen liver samples were available. Nonetheless, these subcohorts were representative from the whole series since statistical analyses ruled out remarkable differences regarding age, sex BMI and AC (data not shown).

Table 2. Prevalence of NAFLD in patients (CAD-SCD group) vs in controls (non-CAD-SD group)

	CAD-SCD group (*N* = 133) *N* (frequency)	Non-CAD-SD group (*N* = 106) *N* (frequency)	OR (95% CI)	*P*
Non-NAFLD	50 (0.38)	78 (0.74)	1*	
NAFLD	83 (0.62)	28 (0.26)	4.62 (2.65–8.07)†	<0.001
			2.63 (1.31–5.28)‡	0.006
Simple steatosis (yes)	27 (0.20)	16 (0.15)	2.63 (1.29–5.37)†	0.038
			1.92 (0.84–4.43)‡	0.123
NASH (yes)	56 (0.42)	12 (0.11)	7.28 (3.55–14.92)†	<0.001
			4.42 (1.67–11.65)‡	0.003
NASH 1	4 (0.03)	3 (0.028)	2.08 (0.45–9.69)†	0.351
			1.34 (0.16–11.18)‡	0.788
NASH 2	37 (0.28)	7 (0.065)	8.25 (3.41–19.93)†	<0.001
			4.94 (1.61–15.18)‡	0.005
NASH 3	15 (0.11)	2 (0.018)	11.70 (2.57–53.36)†	0.001
			13.43 (1.49–121.39)‡	0.021

*Reference group.
†Univariate analysis.
‡Multivariate analysis adjusted for age, sex, body mass index and abdominal circumference.
NASH score as previously reported (NASH 1 mild, NASH 2 moderate and NASH 3 severe) (24).

Patients exhibited higher miR-34a-5p and lower miR-122-5p levels than controls ($P < 0.001$ and <0.01, respectively, Fig. 1A). Furthermore, hepatic miR-34a-5p was statistically increased in patients even in the absence of NAFLD ($P < 0.01$, Fig. 1B).

Expression of hepatic miRNAs according to the severity of the liver disease

Considering pooled data from CAD-SCD and non-CAD-SD individuals, the presence of NAFLD was associated with altered miRNA levels, namely increased miR-34a-5p and decreased miR-21-5p, -29c-3p, -33a-5p and -122-5p levels (Fig. S1A). However, little impact in this unusual miRNA expression associated with NAFLD was observed with respect to the absence/presence of CAD with the exception of a global increase in miR-34a-5p levels (relative expression 1.0–1.3 in Fig. 2A vs 1.8–2.1 in Fig. 2C respectively). When the severity of the liver disease (no NAFLD, steatosis vs NASH) was analysed a trend towards more deregulated miRNAs in more affected livers was observed for several miRNAs (Figs 2B,D and S1B). Moreover, significant direct (for miR-34a-5p) and inverse (for miR-21-5p, -29c-3p and -122-5p) correlations with the severity of the liver disease were observed, with a significant overlap though (Fig. 3).

Correlations between miRNA expression and cardiovascular risk factors

Tables S1, S2 and Fig. S2 show the correlation analyses between miRNAs levels and quantitative continuous risk factors. miR-29c-3p and miR-122-5p showed a significant inverse correlation with obesity parameters (BMI and AC) both among controls and among patients. However, miR-34a-5p did not correlate with those variables and, instead, it exhibited a direct correlation with age and GGT levels ($r = 0.460$, $P = 0.016$ and $r = 0.598$, $P = 0.002$, respectively) only in controls. An unfavourable lipid profile correlated with several miRNAs being the most interesting findings an inverse correlation between miR-122-5p and T-Ch in controls ($r = -0.458$, $P = 0.025$) and an inverse correlation between miR-122-5p and LDL-ch/HDL-ch ratio ($r = -0.379$, $P = 0.016$) in patients. Finally, with respect to inflammation, a significant direct correlation was noted among patients between hs-CRP levels and miR-34a-5p ($r = 0.492$, $P < 0.001$).

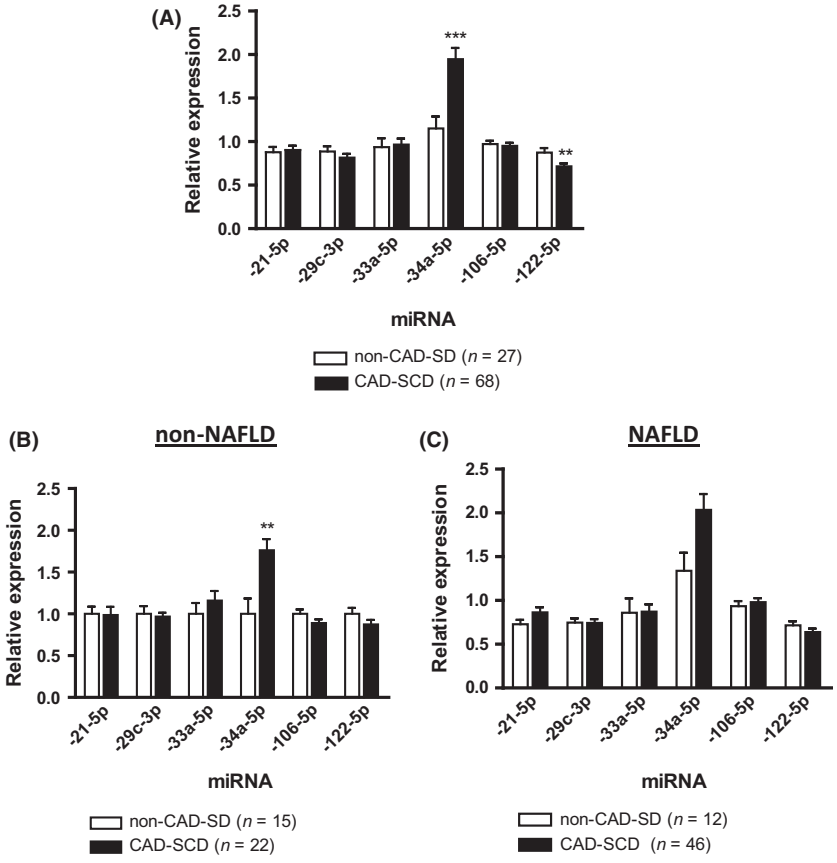

Fig. 1. Comparison of hepatic miRNA expression regarding the presence of CAD in the total group (A) and in the subgroups regarding the absence (B) or presence (C) of NAFLD. $^{**}P < 0.01$, $^{***}P < 0.001$.

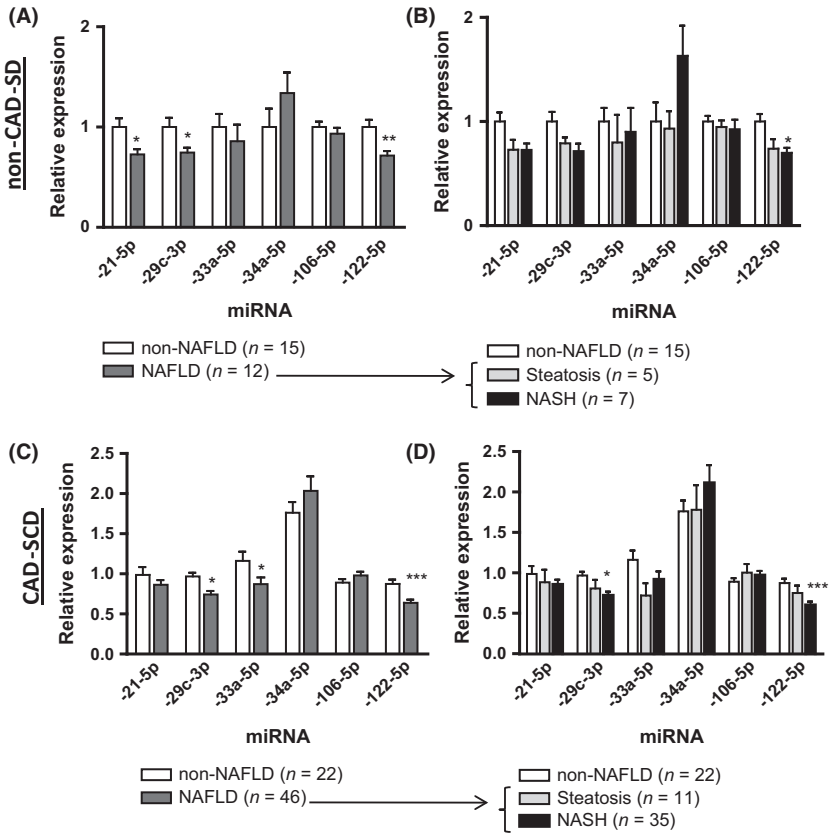

Fig. 2. Hepatic miRNA expression according to status of the liver disease in patients with CAD (A) and without CAD (C). A detailed analysis dividing the NAFLD patients into steatosis and NASH is provided for CAD-SCD patients (B) and non-CAD-SD individuals (D). *$P < 0.05$. ** $P < 0.01$, ***$P < 0.001$. When more than two subgroups were considered, the Bonferroni post-hoc test was applied and the non-NAFLD subgroup was used as reference for comparisons.

Discussion

This study shows for the first time evidence pointing towards hepatic miRNAs as major actors of the interplay between NAFLD and CAD. Specifically in CAD victims without NAFLD, miRNA-34a-5p is the only one significantly deregulated, suggesting that it may promote CAD even before than NAFLD. To our knowledge, our series is the first one to study the presence of NAFLD in middle-aged adult CAD-SCD victims in whom the role of certain miRNAs has also been investigated.

CAD is significantly associated with NAFLD in our study, particularly with more severe liver damage (NASH with higher scores). In keeping with us, similar results have been recently made in clinical series where the diagnosis of NAFLD was based on blood tests and ultrasound imaging without histological confirmation (25–27). NAFLD has also been associated to atherosclerosis in other territories, such as in carotid arteries, where this association was independent of the presence of the classical cardiovascular risk factors (28). In line with most studies (29), we found no differences regarding the prevalence of steatosis among patients and controls in the multivariate analysis of our study, thus reflecting that simple steatosis without inflammation or fibrosis is not a marker of CAD. The scarce available forensic epidemiological data to this respect come from adult forensic autopsies in Iran and Greece, where not only NAFLD and NASH associated to CAD in adults (21, 22) but also to aortic atherosclerosis in children (30).

It has been published that several miRNAs regulate cholesterol homoeostasis and fatty acid metabolism and are implicated in CAD, NAFLD and NASH (17, 31–34). Indeed, mice with diet-induced NASH exhibited a characteristic aberrant hepatic miRNAs pattern similar to our results, namely overexpressed miR-34a, miR-155, miR-200b and miR-221 and downexpressed miR-29c, miR-122, miR-192 and miR-203 (35). Herein we report, for the first time in humans, the correlation of the severity of NAFLD with high hepatic miR-34a-5p and low miR-122-5p and -29c-3p levels (Fig. 3) and, furthermore, also with the presence of a diseased coronary tree (Fig. 1). We have also observed that some miRNAs seem to be more implicated in the development of NAFLD than of CAD. It is the case of miR-21-5p, -29c-3p or -122-5p whose downregulation is inde-

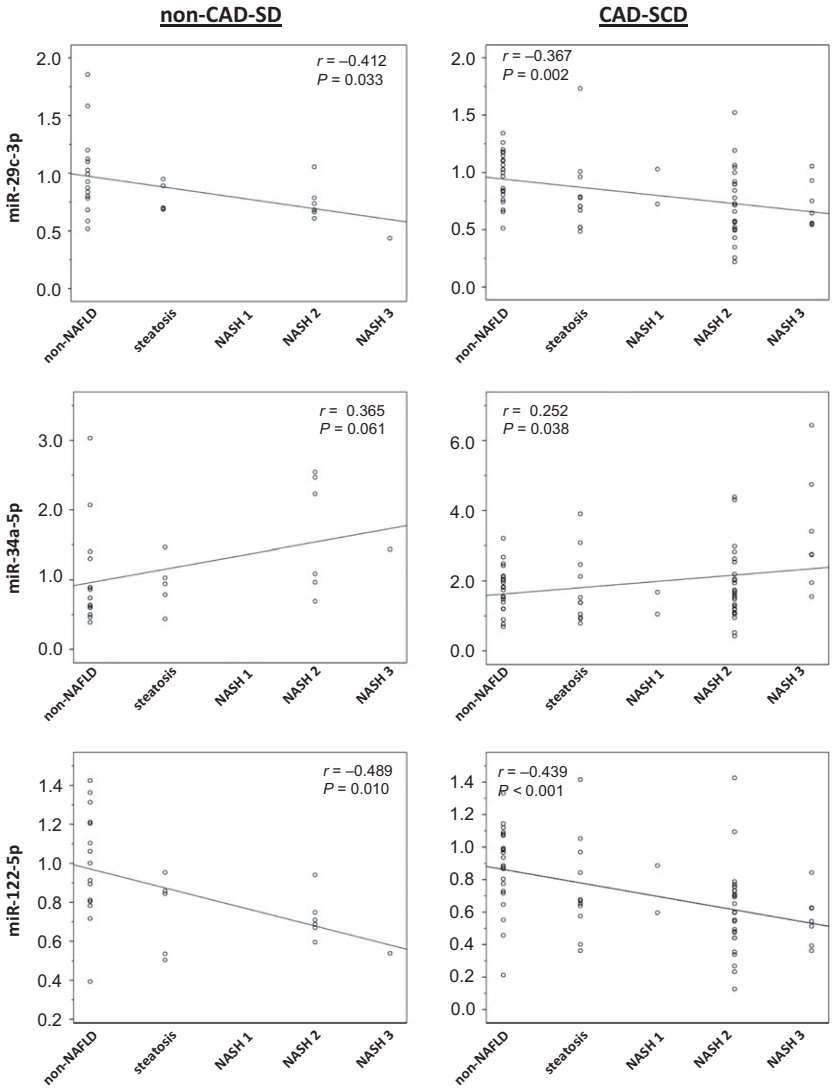

Fig. 3. Correlations between miRNA expression levels and NAFLD scoring.

pendent of the presence of CAD (Figs 1 and 2). In contrast, miR-34a-5p expression seems to be related to CAD irrespective of the liver status (Figs 1 and 2) and also to the presence and severity of NAFLD (Fig. 3). The fact that in CAD patients miR-34a-5p is already upregulated even though no sign of NAFLD is yet observed at the histological evaluation suggests that the hepatic production of this miRNA may precede the coronary burden. Although the mechanism by which this and other hepatic miRNAs may exert their action in the coronary walls remains largely unknown, several tantalizing hypotheses arise. First, the deregulated hepatic miRNAs could reach the blood stream and exert a direct proatherosclerotic effect on the coronary walls. And second, dyslipidaemia and systemic inflammation may indirectly mediate their effect in CAD promotion, provided the deregulated miRNAs influence in the metabolism of cholesterol and proinflammatory citokines before CAD is established. The more relevant associations to this respect include inverse correlations between miR-122-5p and total cholesterol in controls and LDL-Ch/HDL-Ch in patients and also between miR-34a-5p and hs-CRP in patients (Table S2 and Fig. S2). Further studies will be necessary to test these hypotheses.

It has been described that miR-34a-5p, through the SIRT1/p53 pathway, correlate with the degree of NAFLD severity (16) and decreases the phosphorylation of the 3-hydroxy-3-methyl-glutaryl-CoA reductase (HMGCoA), so that the active dephosphorylated form positively influences in the hepatic cholesterol accumulation (16, 36). In our cohort, we have found significant correlation between hepatic miR-34a-5p levels and age in the control group, in keeping with previous reports linking high miR-34a levels with the ageing heart (37). However, miR-34a-5p levels did not significantly corre-

late with age among patients, thus suggesting that in pathological conditions other mechanisms disturb the linearity of that upregulation.

miR-122-5p represents about 70% of total hepatic miRNAs. Controversial results have been published regarding its role in NAFLD. Indeed, we have observed a significant decrease in hepatic miR-122-5p in patients with NAFLD. In agreement with our results, it has been published that it targets hepatic lipogenic genes (16), its hepatic levels inversely correlate with NAFLD or NASH in rats (16, 38, 39) and with NASH in patients (17). Moreover, miR-122-5p silencing reproduced a pattern of hepatic lipogenic mRNA and protein expression that was similar to that observed in human NASH (17). Conversely, other studies have reported that the transient inhibition of miR-122-5p in mice protects against dyslipidaemia (40, 41) and liver steatosis (17, 41). These contradictory results may indicate that it is necessary a long-term miR-122-5p suppression for establishing a liver pathology (17).

Although a deregulated miR-33a-5p expression has been associated to an altered lipid metabolism (42, 43), we could not confirm this point. Moreover, no statistical differences were found in its hepatic levels regarding the presence or absence of CAD (data not shown).

Finally, several papers associated miR-29c-3p hepatic levels with cancer (44, 45) but none have linked this miRNA to CAD, although this association in the herein presented results was weak and only in the extreme groups.

Nowadays, how and when the awareness of NAFLD as a cardiovascular risk factor should be implemented in the clinical arena (in terms of CAD screening) remains to be elucidated. On the contrary, its assessment in the follow up of already diagnosed CAD patients may not be considered suitable since no specific therapeutic approach is warranted for NAFLD.

In terms of *future* daily practice, a new era could be nearby since, as Cecarelli *et al.* suggested (16), the identification of a specific miRNA profile for each stage of NAFLD would represent an important breakthrough for the design of new potential diagnostic or therapeutic tools and might even prevent the development of CAD in NAFLD patients.

In conclusion, the SCD potentially attributable to CAD is associated with a higher prevalence of NAFLD (and NASH) when compared with SD without CAD. The hepatic miRNAs studied appear to be associated to dyslipidaemia, obesity, systemic inflammation and NAFLD severity, being NASH but not steatosis associated to the presence of CAD.

Limitations

We acknowledge that the absence of systematic data regarding classical cardiovascular risk factors in the autopsy files precluded their inclusion in the multivariate analyses. However, their retrospective collection from the relatives is not always amenable and/or accurate. Remarkably, other papers in living patients did systematically check them with similar results in different scenarios. These two considerations blunt this limitation and let us underscore the novelty of our herein reported results in victims of SD. The wide range of time between the last meal and the moment of the exitus on one hand, and the fact that the postmortem lipid profile has not been yet standardized on the other, warrant caution when analysing the correlations with these parameters. Finally, further evidence with a bigger sample size is needed to corroborate our results and elucidate the role of hepatic miRNAs in CAD.

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Supporting information

Additional Supporting Information may be found at onlinelibrary.wiley.com/doi/10.1111/liv.13097/suppinfo

Annex 2. Supplementary Tables

Annex 2: Supplementary Tables

Supplementary Table 1: miRNA microarray expression profiles in control and eutopic endometria OMAs. 157 mature miRNAs differentially expressed (±1.3-FC, p <0.05) in 4 ovarian endometrioma and 7 eutopic endometrial samples (79 up-regulated and 78 down-regulated) compared to the miRNAs in 5 control endometrial samples. Shaded lines indicate miRNA selected for the PCR experiments. (Braza-Boïls et al, 2014)

	OMA *vs* CNT	**EUT *vs* CNT**	**OMA *vs* CNT**	**EUT *vs* CNT**
miRNA	***p***	***p***	**FC**	**FC**
let-7g-star	1.41E-08	0.253971	-1.42043	1.12644
miR-105	3.85E-08	0.373833	-3.64101	-1.124
miR-106b-star	6.00E-08	0.864443	-1.34257	-1.01817
miR-10a-star	1.35E-07	0.117093	-2.88701	1.27307
miR-10a	2.38E-07	0.608	-1.55057	1.02357
miR-10b-star	3.01E-07	0.224114	1.31682	1.06047
miR-1201	5.20E-07	0.0484675	-1.0245	1.36909
miR-1202	7.46E-07	0.138974	-1.38	-1.17143
miR-1224-3p	8.65E-07	0.0095555	-1.32695	-1.30045
miR-1225-5p	1.35E-06	0.418804	-1.31924	-1.06358
miR-1231	2.34E-06	0.228693	-1.34544	-1.13427
miR-1237	2.85E-06	0.192177	-1.39327	-1.18206
miR-1258	3.92E-06	0.869168	1.41744	1.01806
miR-1260	1.18E-05	0.173522	-1.35304	-1.10788
miR-1273c	1.89E-05	0.0883607	-1.10595	1.36071
miR-1279	2.03E-05	0.751458	1.65594	1.08452
miR-1295	3.95E-05	0.690758	2.8169	1.1714
miR-1296	4.09E-05	0.990743	-1.39942	1.00096
miR-1307	5.18E-05	0.522256	-1.41382	-1.07013
miR-133a	7.54E-05	0.929811	2.26086	-1.02773
miR-133b	8.26E-05	0.955377	1.93306	-1.01498
miR-134	9.59E-05	0.674239	1.31171	1.01441
miR-137	0.0001107	0.122963	-1.31089	-1.15996
miR-138	0.0001203	0.0285628	1.14975	1.49604
miR-139-3p	0.0001323	0.0412209	2.36231	1.22432
miR-139-5p	0.0001343	0.639812	1.4366	1.0323
miR-141	0.0002003	0.906475	-4.4174	1.00734
miR-145-star	0.0002385	0.326091	1.70673	1.23299
miR-148b-star	0.0002872	0.145969	-1.42315	-1.16203
miR-150-star	0.0004047	0.373888	-1.3224	-1.08928
miR-154-star	0.0005433	0.279797	2.11116	1.34667
miR-154	0.000547	0.444458	1.30144	1.08765
miR-15a-star	0.0006903	0.0892655	-1.48196	-1.2461
miR-16	0.00073	0.772305	-1.06806	1.00307
miR-182	0.000976	0.776912	-1.53864	1.01487
miR-183-star	0.001051	0.902082	-2.25569	-1.01673

Supplementary Table 1: *Continued*

	OMA *vs* CNT	**EUT *vs* CNT**	**OMA *vs* CNT**	**EUT *vs* CNT**
miRNA	***p***	***p***	**FC**	**FC**
miR-183	0.0010513	0.540128	-2.31699	1.06526
miR-187-star	0.0011347	0.805025	-1.88765	1.03608
miR-187	0.0012267	0.753743	-2.2859	1.02637
miR-18a-star	0.0013091	0.674894	-1.53722	1.03141
miR-18b	0.0016365	0.935189	-1.51142	1.00577
miR-191-star	0.0019191	0.828487	-1.51766	-1.0264
miR-1913	0.0019936	0.121841	-1.32348	-1.17186
miR-196b-star	0.0022193	0.794653	-3.50485	-1.01808
miR-196b	0.0022818	0.906985	-2.24398	1.00937
miR-1972	0.0022851	0.259205	-1.33904	-1.09708
miR-199b-5p	0.0023489	0.576408	-1.55621	1.03307
miR-1	0.0023565	0.829432	2.07514	-1.06445
miR-200a-star	0.0024576	0.401051	-3.42394	1.04233
miR-200a	0.0025697	0.740816	-2.96719	1.01977
miR-200b-star	0.0026123	0.593033	-3.01642	1.0344
miR-200b	0.0026686	0.398275	-1.63369	1.06509
miR-200c-star	0.0027052	0.228852	-3.10427	1.15808
miR-200c	0.0033242	0.737249	-1.35786	1.01611
miR-202	0.0033875	0.496386	6.16021	1.11787
miR-203	0.0033991	0.304762	-1.30548	1.05361
miR-205	0.0035134	0.77669	-3.13537	-1.04692
miR-20b-star	0.0036635	0.926652	-2.43251	-1.01709
miR-2115	0.0037713	0.365126	1.56062	-1.16006
miR-217	0.0041206	0.871441	2.15271	-1.08911
miR-26a-1-star	0.0048873	0.681856	1.33025	-1.03802
miR-27a-star	0.004961	0.869728	-1.39722	-1.01807
miR-29b-2-star	0.0056248	0.0868307	1.38267	1.1071
miR-29b	0.0056952	0.0720577	1.473	1.30412
miR-29c-star	0.0057425	0.83455	1.61682	1.03901
miR-29c	0.0058844	0.0998617	1.52029	1.19676
miR-301a	0.0058887	0.372941	-1.38965	1.08215
miR-3128	0.0065399	0.119471	-1.69255	-1.18195
miR-3130-5p	0.0067339	0.0304271	-1.43426	-1.27128
miR-3131	0.0067827	0.676173	-1.90944	-1.05369
miR-3146	0.0071528	0.357535	1.31336	-1.1005
miR-3167	0.0072235	0.0199481	1.21542	1.32074
miR-3180-5p	0.0079509	0.119937	-1.42625	-1.2309
miR-3181	0.0079745	0.130963	-1.60049	-1.25456
miR-3187	0.0088328	0.428774	-1.4498	-1.10494
miR-323-3p	0.0101744	0.446395	1.50883	1.1374
miR-329	0.0101908	0.0804762	1.82938	1.4622
miR-337-5p	0.0103706	0.0440539	1.33396	1.06103

Supplementary Table 1: *Continued*

	OMA *vs* CNT	EUT *vs* CNT	OMA *vs* CNT	EUT *vs* CNT
miRNA	***p***	***p***	**FC**	**FC**
miR-33b-star	0.0107608	0.934205	-1.54723	-1.00976
miR-34b-star	0.0120587	0.58557	-3.37599	1.04647
miR-34c-3p	0.0121033	0.843182	-1.86381	-1.01182
miR-34c-5p	0.012113	0.885242	-2.54921	1.00834
miR-363	0.0124559	0.676924	-1.90897	1.04177
miR-365-star	0.0124795	0.496798	-1.74047	-1.08676
miR-369-5p	0.0130217	0.205946	1.72363	1.25363
miR-373-star	0.0136627	0.0108726	-1.59872	-1.5995
miR-375	0.013883	0.159313	-7.32669	1.11089
miR-376a	0.0143692	0.295939	1.79812	1.2785
miR-376b	0.0144752	0.444027	1.87265	1.21318
miR-376c	0.0146907	0.0506156	1.36655	1.0816
miR-377	0.0146907	0.590288	1.72118	1.13438
miR-379-star	0.015173	0.973202	1.38807	1.00541
miR-379	0.015309	0.413468	1.31144	1.02579
miR-380-star	0.0158033	0.0226606	1.40908	1.41178
miR-380	0.0158954	0.120951	1.40519	1.25951
miR-381	0.0161666	0.330255	1.52236	1.06506
miR-382	0.0168578	0.171206	1.33225	1.04987
miR-383	0.0169567	0.92529	2.4162	-1.02326
miR-410	0.0173802	0.258552	1.87887	1.33119
miR-411-star	0.0173826	0.0198753	2.41509	1.73618
miR-411	0.0177904	0.208284	1.3027262	1.0831
miR-424	0.0190415	0.178638	1.13317	1.32434
miR-4259	0.0201184	0.216258	-1.65207	-1.24131
miR-4260	0.0209709	0.0106697	-1.56631	-1.28469
miR-4266	0.0210268	0.238715	1.46523	1.16699
miR-4269	0.021359	0.589693	1.32746	1.02478
miR-4282	0.0215459	0.784338	1.32762	1.03134
miR-4289	0.0220109	0.0041093	1.38842	1.58042
miR-4291	0.0221256	0.469978	1.34519	1.09579
miR-4295	0.0225268	0.0040281	1.39765	1.34234
miR-429	0.0226203	0.199549	-1.99696	1.17789
miR-4304	0.0229108	0.892468	-1.71388	1.01949
miR-4310	0.023066	0.6409	-1.64012	-1.0552
miR-4312	0.0232157	0.0207375	-1.70635	-1.34343
miR-4321	0.0235959	0.0717746	-1.43315	-1.27586
miR-4322	0.0237082	0.587528	-1.44199	-1.06234
miR-4323	0.0237354	0.142816	1.35823	1.20499
miR-4324	0.0249906	0.766789	1.48604	1.04715
miR-4327	0.0259863	0.383906	-1.7141	-1.15213
miR-4328	0.0260796	0.813804	1.35579	1.02701

Supplementary Table 1: *Continued*

	OMA *vs* CNT	EUT *vs* CNT	OMA *vs* CNT	EUT *vs* CNT
miRNA	***p***	***p***	**FC**	**FC**
miR-4329	0.0268521	0.692055	1.37956	-1.03383
miR-449a	0.0273232	0.947483	-6.2269	1.00481
miR-449b-star	0.0284596	0.408264	-2.10914	-1.09801
miR-449b	0.0289221	0.864427	-5.62438	1.01132
miR-449c	0.0291551	0.75029	-6.65633	-1.02842
miR-455-5p	0.0296142	0.526749	-1.45645	1.0542
miR-485-5p	0.031029	0.125545	1.61538	1.11701
miR-487a	0.0311761	0.71624	1.30757	1.02871
miR-488-star	0.0314188	0.369756	1.73522	1.18705
miR-493-star	0.0320114	0.417161	2.10393	1.25816
miR-495	0.0323823	0.577055	1.40007	1.08611
miR-498	0.0340514	0.354413	-1.69468	-1.17826
miR-506	0.0352368	0.995806	5.43785	1.00521
miR-508-3p	0.0353955	0.782699	4.63775	-1.26114
miR-508-5p	0.0354128	0.815646	4.35959	-1.20533
miR-509-3-5p	0.0354752	0.746023	4.72703	-1.23775
miR-509-3p	0.0359608	0.893988	3.28523	-1.03633
miR-509-5p	0.0367118	0.95553	5.9795	1.05478
miR-510	0.0369986	0.875238	5.65882	1.10648
miR-513a-5p	0.0386238	0.598215	2.6241	1.22202
miR-513b	0.0391217	0.854393	3.15697	1.09547
miR-513c	0.0394433	0.756224	5.83939	1.27952
miR-514	0.0406085	0.898231	3.1498	1.08116
miR-514b-3p	0.0409719	0.99455	3.26352	1.00399
miR-514b-5p	0.0430791	0.675491	3.92318	-1.11671
miR-515-5p	0.0433026	0.842883	1.39418	1.03247
miR-517b	0.0437095	0.872849	1.37707	1.02318
miR-518c-star	0.0438257	0.293337	1.61077	1.21185
miR-518d-5p	0.0438974	0.0120982	1.32727	1.32389
miR-520d-5p	0.0466534	0.445333	1.49653	-1.11468
miR-524-5p	0.049869	0.724032	1.60388	1.06684
miR-539	0.0499675	0.582719	2.54076	1.20587
miR-556-3p	0.0669232	0.0011172	-1.37746	-1.42387
miR-578	0.088775	0.0053565	1.29228	1.57777
miR-636	0.128153	0.0488759	-1.76706	-1.52172
miR-892b	0.475989	0.0114275	-1.01557	-1.36867
miR-935	0.848006	0.0154318	-1.68699	-1.4834

Supplementary Table 2: miRNA microarray expression profiles in eutopic cells in response to EPF. 82 mature miRNAs differentially expressed (±2.0-FC, p <0.05 and) in response to EPF of eutopic cells in comparison to the same cell culture with ØPF exposition (72 down-regulated and 10 up-regulated). Shaded rows indicate miRNA selected for the PCR experiments. (Braza-Boïls et al, 2015)

miRNAs down-regulated	*p*	FC
miR-20a	0.0111598	- 21.4718064
miR-106b	0.0100192	- 20.3463353
miR-185	0.0124794	- 19.8490677
miR-130a	0.0051569	- 19.2517981
miR-19b	0.0094816	- 17.9645774
miR-21	0.0062025	- 16.9641903
let-7i	0.0167231	- 15.9376265
miR-130b	0.0143087	- 12.9471794
miR-324-5p	0.0137468	- 11.8440891
miR-27a	0.0285212	- 11.0489668
miR-379	0.0321505	- 10.9900496
miR-106a	0.0250609	- 10.7467486
miR-143	0.0225156	- 10.3996481
miR-16	0.0432001	- 9.96869829
miR-152	0.0223745	- 9.40432975
miR-18a	0.0279132	- 9.18644815
miR-28-5p	0.0111490	- 8.83970086
miR-542-5p	0.0183053	- 7.73945886
miR-487b	0.0195141	- 7.06578957
miR-210	0.0212674	- 6.92429666
miR-625	0.0371068	- 6.84537663
miR-151-3p	0.0278055	- 6.76448106
miR-30a-star	0.0358941	- 6.42421673
miR-21-star	0.0088882	- 6.38316886
miR-382	0.0162315	- 6.21160452
miR-17	0.0202613	- 6.13109507
miR-500-star	0.0141254	- 6.03278214
miR-20b	0.0282727	- 5.70138430
miR-143-star	0.0072587	- 5.69336666
miR-214-star	0.0125762	- 5.41230976
miR-532-5p	0.0408379	- 5.32864413
miR-652	0.0326773	- 5.32246132
miR-503	0.0367789	- 5.30062494
miR-199b-3p	0.0438447	- 5.27445634
miR-30b	0.0111379	- 5.18360323
miR-195	0.0031788	- 4.99004486
miR-27b	0.0455584	- 4.85173110
miR-431	0.0349890	- 4.85086030
miR-708	0.0305980	- 4.81549433
miR-376c	0.0452613	- 4.48360123
miR-29a	0.0249246	- 4.32883567
miR-93-star	0.0365464	- 4.03952269
miR-584	0.0484412	- 3.82130069
miR-22	0.0279155	- 3.82099407
miR-22-star	0.0214495	- 3.68038099
miR-409-5p	0.0494708	- 3.39721225
miR-299-3p	0.0308237	- 3.34706747

Supplementary Table 2: *Continued*

miRNAs downregulated	*p*	FC
miR-381	0.0019607	- 3.28317733
miR-24	0.0182050	- 3.13410850
miR-107	0.0400942	- 3.11537155
miR-4321	0.0459466	- 2.99226499
miR-103	0.0393397	- 2.97411037
miR-4317	0.0396322	- 2.88691937
miR-769-5p	0.0317719	- 2.86410673
miR-224	0.0191904	- 2.59635731
miR-1287	0.0333251	- 2.59237272
miR-26a	0.0376347	- 2.56181007
miR-758	0.0060906	- 2.53725966
miR-18b	0.0207590	- 2.48097095
miR-34a-star	0.0483251	- 2.44714174
miR-200c	0.0174579	- 2.42423655
miR-125b-2-star	0.0413748	- 2.40571405
miR-29c	0.0130110	- 2.38095238
miR-887	0.0336026	- 2.24207706
miR-335	0.0356932	- 2.21417738
miR-660	0.0211060	- 2.20873910
miR-301a	0.0103374	- 2.08830611
miR-181a-star	0.0474907	- 2.04848770
miR-424	0.0471200	- 2.03837859
miR-221	0.0394826	- 2.02963264
miR-151-5p	0.0434842	- 2.02071638
miR-874	0.0167659	- 2.00180563
miRNAs up-regulated	***p***	**FC**
miR-197	0.0426825	5.20828
miR-1975	0.0040120	5.14247
miR-138-1-star	0.0387331	4.66479
miR-886-3p	0.0179947	3.55023
miR-149	0.0476597	3.43946
miR-4298	0.0212579	3.43411
miR-1207-5p	0.0306547	3.20857
miR-3175	0.0334685	3.05652
miR-320d	0.0215316	2.68635
miR-574-3p	0.0155365	2.43088

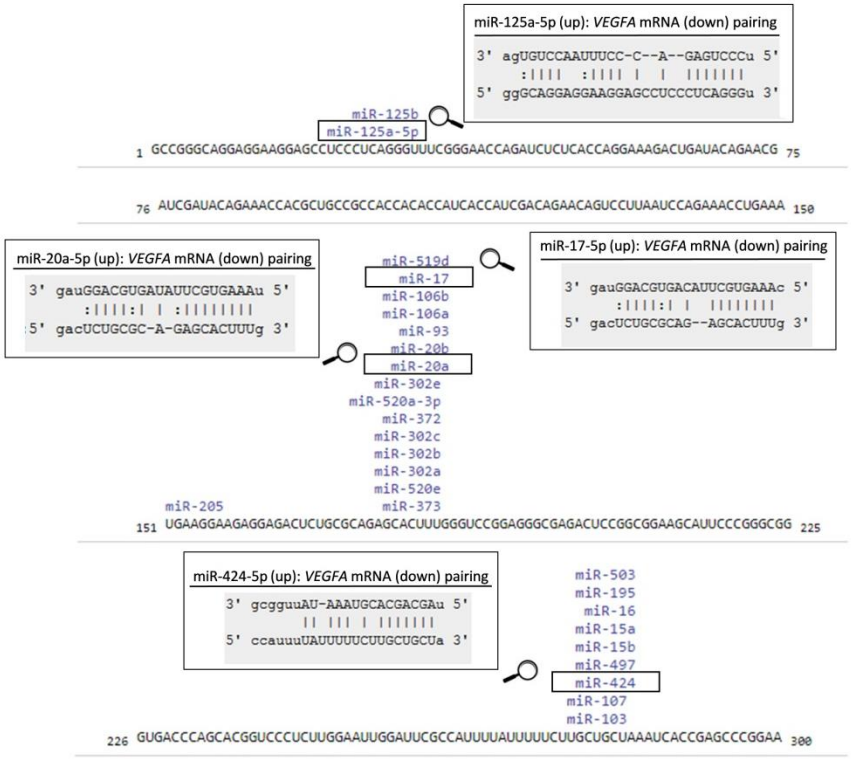
Supplementary Table 3: Annotation of the 11 CpG analysed by bisulphite sequencing.

CpG number	Nucleotide position from CpG nº1	Position within amplicon	Genomic localization
1	1	23	43738195
2	6	28	43738200
3	9	31	43738203
4	103	125	43738297
5	118	140	43738312
6	133	155	43738327
7	135	157	43738329
8	137	159	43738331
9	141	163	43738335
10	145	167	43738339
11	152	174	43738346

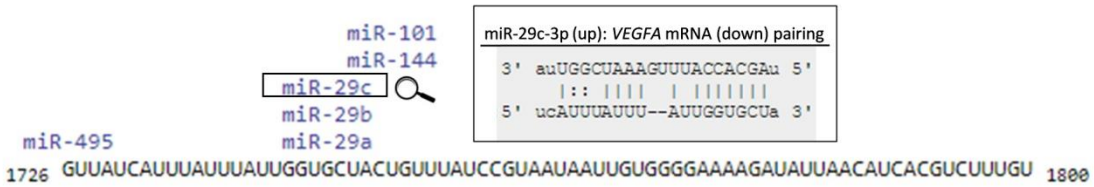
Annex 3. Supplementary Figures

Annex 3: Supplementary Figures

Supplementary Figure 1. *VEGFA* mRNA 3'-UTR: selected miRNA pairing. Nucleotides from 1 to 300 are represented. Watson – Crick pairing for *VEGFA* 3'-UTR and the differently expressed miRNAs miR-125a-5p, miR-17-5p, miR-20a-5p and miR-424-5p are represented in boxes. Data has been obtained from microRNA.org database (http://www.microrna.org/).



Supplementary Figure 2. *VEGFA* mRNA: miR-29c-3p pairing. 3'-UTR of *VEGFA* (from nt 1726 to 1800) is represented. Watson – Crick pairing for *VEGFA* 3'-UTR: miR-29c-3p pairing is represented in the box. Data has been obtained from microRNA.org database (http://www.microrna.org/).



Supplementary Figure 3. Time-dependent increase of HEK-293 GFP+ cells after FuGene® HD transfection. Transfection were performed with with plasmids for Dnmt3a, specific ZFP for *VEGFA* promoter and GFP (as reporter gene), employing the FuGene® HD transfection reagent (Promega). Upper row: optical microscopy images. Lower row: fluorescence microscopy images (Scale 1:31).