Circulating microRNAs in endometriosis

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ABSTRACT

Endometriosis is defined as the presence of endometrial tissue outside the uterus and is the leading cause of disability in reproductive-age women. The pathogenesis of endometriosis remains unclear and no blood test is available for early diagnosis of the disease. MicroRNAs (miRNAs) are endogenous small ribonucleic acids (RNAs) that have important gene-regulatory roles via posttranscriptional suppression of target genes. The biological importance of miRNAs, initially demonstrated in cancer, has more recently been confirmed in other diseases. In light of the sustained presence of miRNAs in the circulation and given the emerging evidence on aberrant miRNA expression in endometriotic tissue, we hypothesized that endometriosis is associated with unique plasma miRNA signatures that have diagnostic potential and possibly contribute to disease pathogenesis.

In order to test this hypothesis, we established a procedure to measure plasma miRNAs and created a tissue bank of prospectively collected blood and eutopic endometrium samples. Next, by using qRT-PCR-based arrays we screened the plasma of a small set of women (n = 16) with or without endometriosis for miRNA content at 3 different phases of the menstrual cycle. We demonstrated that plasma miRNAs do not fluctuate across the cycle and identified 12 plasma miRNAs that are differentially abundant in endometriosis. In silico functional analyses revealed that these miRNAs and their predicted targets have functional relevance in endometriosis, being involved in molecular pathways known to be associated with the disease.

Using a microarray methodology, we profiled miRNAs in eutopic endometrium from women with and without endometriosis. We demonstrated no correlation between dysregulated miRNAs in endometrium and plasma, suggesting that the differentially abundant circulating miRNAs are not released from the endometrium. Mir-551a and mir-148a* were significantly dysregulated in the endometrium from women with endometriosis, and thus are putative diagnostic markers and therapeutic targets. We also identified differences in miRNA expression between endometriosis-free women with and without pelvic pain, suggesting that pelvic pain might independently modify the endometrial miRNA profile.

Finally, we assessed the value of plasma miRNAs as molecular markers for endometriosis in a prospective diagnostic study in a larger cohort (n = 68) of symptomatic women by using singleplex qRT-PCR. The diagnostic accuracy of circulating miRNAs for patients with

endometriosis was assessed with a predictive algorithm incorporating miRNA expression levels and clinical parameters. A model that included mir-155, mir-574-3p, mir-133a and mir-30c, history of infertility and previous miscarriages demonstrated an accuracy of 84.1% with 93.5% sensitivity, 58.8% specificity and AUC = 0.831.

This thesis presents potential novel biomarkers for early detection of endometriosis, laying the ground work for future efforts to develop blood-based biomarkers for this disease. An accurate non-invasive test would reduce the need for a surgical diagnosis, would be more accessible to women, and is likely to lead to an earlier diagnosis and treatment of endometriosis. Our results need to be confirmed in larger independent patient groups in different populations. In addition, this work raises the possibility that plasma miRNAs may provoke some of the adverse health epiphenomenon associated with endometriosis, which potentially could be altered by therapeutic manipulations of endometriosis-associated plasma miRNAs. Future studies and broader miRNA profiling may elucidate a relationship between miRNAs, endometriotic disease and its severity.

DECLARATION

This work contains no material which has been accepted for the award of any other degree or diploma at any university or other tertiary institution and, to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference has been made in the text.

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PUBLICATIONS ARISING FROM THIS THESIS

Non-invasive tests for the diagnosis of endometriosis. Protocol for the Cochrane review.

Nisenblat V, Farquhar C, Akoum A, Fraser I, Bossuyt PMM, Hull ML.

The Cochrane library, January 2012

Plasma miRNA profiles in women with and without endometriosis – additional considerations towards development of a miRNA-based blood test.

Nisenblat V, Print C, Evans S, Ohlsson-Teague EMC, Robertson S, Hull ML.

Submitted for review, Human Reproduction, January 2013.

Role of miRNAs in endometrial disease in association with reproductive disorders and prospects for circulating miRNAs in diagnosis of endometrial function.

Nisenblat V, Hull ML.

RBM Online, in press 2013.

COMMERCIAL POTENTIAL ARISING FROM THIS THESIS

Patent

Method for identifying endometriosis.

Key contributors: Nisenblat V, Print C, Robertson SA, Ohlsson Teague EMC, Hull ML.

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Funding for further work

This thesis served a basis for successful grant application for further work to develop a validated prototype blood test for diagnosis of endometriosis.

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ABSTRACTS ARISING FROM THIS THESIS

Menstrual cycle variations in plasma microRNA expression profiles

Ohlsson Teague EMC, Nisenblat V, Robertson SA, Hull ML

Poster presentation. Presented at SRB conference on 23-26.08.2009, Adelaide, Australia.

A unique plasma microRNA expression profile is identified in women with endometriosis

Nisenblat V, Print CG, Evans S, Ohlsson Teague EMC, Robertson SA, Hull ML.

Poster presentation. Presented at the 11th World Congress of Endometriosis on 4-7.09.2011, Montpelier, France.

Plasma miRNAs as non-invasive biomarkers for endometriosis

Nisenblat V, Robertson SA, Evans SF, Hull ML.

Poster presentation. Presented at the ASRM annual meeting on 22-26.10.2012, San Diego, USA.

Circulating microRNAs as potential biomarkers for endometriosis.

Nisenblat V, Wang Z, Robertson SA, Evans SF, Hull ML.

Oral presentation. Presented at Fertility Society of Australia (FSA) conference 28-31.10.12, Auckland, NZ.

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"…I'm just a normal woman except that I have a horrifying disease called endometriosis. Every month my cycles seem to get worse, making work difficult. I don't enjoy calling in sick and being perceived as being unreliable. I suffer from migraines daily, along with fatigue and pelvis/ back pains … and I never feel well. I feel like its one thing after another and I'm tired of seeing so many doctors. I'm taking 8 pills a night and I'm only 19. When does it end!!! This has to be one of the most frustrating conditions in the world. Peoples reaction seems to be suck it up, it won't kill you be thankful for that. But what kind of life is this?" **Sarah**

"… It took me 3 years for a doctor to finally listen to me. I begged doctors to listen to my symptoms....they all assumed I just wanted the pain-relief pills. I hate depending on anything. I can't do the hormones anymore. I want my life back..." **Hannah**

"My family doesn't quite understand and I've lost almost all of my friends due to the fact that I can't stay out for longer than a couple of hours without feeling drained and ill. My boyfriend of 3 years thinks that I use it as an excuse to not be more active and it's starting to tear us apart. I don't know what to do and I feel completely alone and hopeless at this point. I've lost all hope in the chance that I'll become better ..." **Fiona**

"… The pain has made me jealous of so many things. Jealous of people who don't have pain, jealous of people who have been able to easily start families, jealous of people who have never known what it feels like to have to make the choices I do. Like should I go out tonight or stay home? Should I take these pain killers and feel better but damage my insides even more? Should I eat this now and feel like crap for days after? Sometimes I think jealousy is the worst side effect of this disorder". **Tracey**

(Quotations taken from women diagnosed with endometriosis, 2010-2012)

ABBREVIATIONS

3’-UTR	3’-untranslated regions
AE	amplification efficiency
Ago	argonaute proteins
AID	activation-induced cytidine deaminase
AML	acute myeloid leukaemia
ANOVA	analysis of variance
Anti -2HSG	2 Heremans-Schmidt glycoprotein
ANXA 1	annexin 1
AUC	area under the ROC curve
Bax	BCL2-associated X protein
BCL-2	B-cell lymphoma protein2
B-H	Benjamini-Hochberg method
Bic	B cell integration cluster gene
C. Elegans	Caenorhabditis elegans
Ca-125	cancer antigen 125
CASP	caspase, apoptosis related cysteine peptidase
CCND1	cyclin D1
CDC42	targets cell division cycle 42 protein
CDKN	cyclin-dependent kinase inhibitor
cDNA	complementary DNA
cEBP	CCAAT enhancer binding protein
CK1a	Cysteine kinase 1 alpha
CLL	chronic lymphocytic leukaemia
cMaf	musculoaponeurotic fibrosarcoma oncogene homolog
COL	collagen-matrix proteins
COX	cyclooxygenase
Cq	quantification cycle
CREBBP	CREB binding protein
Ct	cycles to threshold
CTNNB1	β-catenin
CV	coefficient of variance
CXCL	chemokine (C-X-C motif) ligand
CYP19A1	cytochrome P450, family 19, subfamily A, polypeptide
CYR61	cysteine-rich, heparin-binding protein
DNA	deoxyribonucleic acid
DNMT	DNA-(cytosine-5-methyltransferase
DUSP5	dual specificity phosphatase 5
E2	oestradiol
E2F8	E2F transcription factor 8
EGF	epidermal growth factor
EGFR	epidermal growth factor receptor
ER	oestrogen receptor
ERBB	v-erb-erythroblastic leukaemia viral oncogene homolog
ERK	extracellular signal-regulated kinase
ESCs	endometrial stromal cells
FADD	Fas associated death domain protein
FC	fold change
FDR	false discovery rate
FGF	fibroblast growth factor
FOXO3	fork head box O3 gene

FSH	follicle-stimulating hormone
GBM	glioblastoma multiforme
GBS	glial cells missing binding site
GEO	gene expression omnibus
GM-CSF	granulocyte macrophage colony-stimulating factor
GNRH	gonadotropin-releasing hormone
HCC	hepatocellular carcinoma
HDL	high-density lipoprotein
HESF	human endometrial stromal fibroblasts
HGF	hepatocyte growth factor
HIF-1α	hypoxia inducible transcription factor -1 alpha
HLA	human leukocyte antigen
ICAM	intercellular adhesion molecule
IFN	interferon
IGF	insulin-like growth factor
IgG	immunoglobulin G
IKB	ingenuity knowledge base
IKKB	inhibitor of kappa light polypeptide gene enhancer in B-cells, kinase beta
IKKέ	IkB kinase epsilon
IL	interleukin
ILGF	insulin-like growth factor
INF	Interferon
IPA	ingenuity systems pathway analysis
IRS-1	insulin receptor substrate-1
JNK	c-Jun N-terminal kinase
KRAS	Kirsten ras oncogene homolog
LDL	low density lipoprotein
LH	luteinizing hormone
LIF	leukaemia inhibitory factor
LIMMA	linear models for microarray analysis
LNA	locked nucleic acid
LOWESS	locally weighted regression and smoothing scatterplots
LPS	Lipopolysaccharide
MAPK	mitogen-activated protein kinase
MAQC	microarray quality control
MCAP	monocyte chemoattractant protein
MCL1	myeloid leukaemia cell differentiation protein
MESDC1	mesoderm development candidate 1
MET- proto-oncogene	MNNG HOS Transforming gene
MGMT	methyl guanine methyl transferase
MIF	migration inhibitory factor
MIP	macrophage inflammatory protein
MIP	macrophage inflammatory protein
MIQE	minimum information for publication of qRT-PCR experiments
miRNA	micro RNA
MMP	matrix metalloproteinase
MRI	magnetic resonance imaging
mRNA	messenger ribonucleic acid
MSC	mesenchymal stem cells
MSH	melanocyte-stimulating hormone
MSP	miRNAs-specific reverse transcription primer
MTMMP	membrane type matrix metalloproteinase
MVB	multivesicular bodies

NFkB	nuclear factor kappaB
ng	nanograms
NK cells	natural killer cells
NP1	nucleoplasmin 1
NPM1	nucleophosmin
NRT	no reverse transcriptase
nSMase2	sphingomyelinase 2
NTC	no template controls
OD	optical density
P4	Progesterone
PAE	percentile amplification efficiency
PAGE	polyacrylamide gel electrophoresis
PCA	principal components analysis
PCOS	polycystic ovary syndrome
PCR	polymerase chain reaction
PDGF	platelet-derived growth factor
PG	prostaglandin
pg	picograms
PGP 9.5	protein gene product 9.5
PP14	serum placental protein/ glycodelin A
PPARα	peroxisome proliferator-activated receptor, alpha
PR	progesterone receptor
pre-miRNA	precursor miRNA
pri-miRNA	primary miRNA
PRL-3	phosphatase regenerating liver-3 gene
PTEN	phosphatase and tensin homolog
qRT-PCR	quantitative reverse transcriptase PCR
QUADAS	quality assessment of diagnostic accuracy studies included in systematic reviews
r_s	Spearman correlation coefficient
rASRM	revised American Society of Reproductive Medicine classification
REST	RE1-silencing transcription factor
RHOA	Ras homolog gene family, member A
RIN	RNA integrity number
RIPK1	receptor interacting serine–threonine kinase1
RISC	RNA-induced silencing complex
RNA	ribonucleic acid
ROC	receiver-operating characteristics
RQI	RNA quality index
RXRα	retinoid X receptor, alpha
SD	standard deviation
sEcadherin	soluble E-cadherin
SEM	standard error of mean
sEselectin	soluble E-selectin
SF	steroidogenetic factor
SHIP-1	Src homology 2-containing inositol phosphatase-1
sICAM	soluble intercellular adhesion molecules
SMAD	Sma- and Mad-related protein
SMRT	single-molecule real-time sequencing
SMS	single-molecule sequencing
SNR	signal-to-noise ratio
SOCS	suppression of suppressor of cytokine signalling
Sox17	sex determining region Y-related HMG box-17

SQSTM1	Sequestome1 gene
StAR	steroidogenic acute regulatory protein
STARD	standards for reporting of diagnostic accuracy
STAT	signal transducer and activator of transcription
sVCAM	soluble vascular cell adhesion molecule
TCF4	transcription factor 4
TCL1	T cell leukaemia/lymphoma 1
TDP-43	TAR-DNA-binding protein-43
TGF	transforming growth factor
Th	T helper cells
TIMP	tissue inhibitors of MMP
TLDA	TaqMan Low Density miRNA array
TLR	Toll-like receptor
TNF	tumour necrosis factor
TP-53	tumour protein p-53
TP-53INP1	TP-53-induced nuclear protein 1
TR4 [NR2C2]	nuclear receptor subfamily 2 group C member 2
TSP-1	thrombospondin 1 angiogenic protein
UPS	ubiquitin proteasome system
US	ultrasound
VEGF	vascular endothelial growth factor
VENTX	VENT homeobox
VIP	vasoactive intestinal peptide
VSN	variance stabilizing normalization
XIAP	X-linked inhibitor of apoptosis protein
ZFP36	zinc finger protein 36

Chapter 1
Introduction

1.1 Structure of the thesis

This thesis investigates the role of circulating plasma miRNAs in women with endometriosis with the intent to establish microRNA (miRNA)-based non-invasive diagnostic test for the disease. The included studies provide insight into circulating miRNAs within the field of endometriosis research and highlight further, more specific areas of investigation by bringing the experimental findings into a clinical context. A brief outline of the studies included in this thesis is presented below.

The background of the study is presented in Chapter 1 and is subdivided into 2 topics. The first topic summarises the available evidence on clinical, epidemiological, social and experimental issues associated with endometriosis. The data presented emphasises the complexity associated with this enigmatic disease and underscores the need in novel approaches to investigate the associated pathogenetic mechanisms and to establish a non-invasive diagnostic test. The second topic focuses on miRNAs, a class of recently discovered regulators of vital cellular functions via complex fine-tuning of gene expression networks. The unique qualities of miRNAs and the fast moving field of molecular diagnostics make these small non-coding RNAs an attractive tool in physiological and diagnostic research. Based on the literature overview and incorporating aspects of clinical relevance, a set of working hypotheses of the involvement of circulating miRNAs in endometriosis was generated.

In order to test the above hypotheses we first overviewed the methodological aspects of miRNA quantification and analysis with emphasis on circulating miRNAs. The detailed discussion on the experimental and analytical methods of miRNA research as well as the gaps and controversies in the existent knowledge in the field is presented in Chapter 2.

Chapter 3 outlines how the experimental methodology was established. The main focus was the initiation of the tissue bank and prospective collection of samples as a source of experimental material. In addition to this, the development of the process for miRNA extraction from plasma samples is described. Finally, revision of the methods for proper design and conduct of the diagnostic accuracy study are documented. This chapter also includes the description of the laboratory techniques and statistical analyses implemented in the project.

Chapter 4 identifies an endometriosis-specific plasma miRNA profile and its dependence on physiological menstrual cycle stage. Further, computational analyses were implemented to

assess the molecular events associated with the set of identified endometriosis-specific miRNAs and their predicted targets.

Chapter 5 examines existing knowledge on the role of identified miRNAs in human disease. This includes the data obtained from functional, comparative and computational studies in various diseases and tissues. The rationale for conducting this review was to assess the functional relevance of these miRNAs in endometriosis. Considering that the identified miRNAs have not been previously documented in association with endometriosis, an assumption was made that specific miRNAs might exert a comparable function in different diseases with similar underlying pathologies. Therefore, pathological processes similar to those known to occur in endometriosis were of the most interest.

Chapter 6 presents a study in which the miRNA profile was examined in secretory eutopic endometrium in women with and without endometriosis and its concordance with the miRNA signature identified in plasma is analysed. The study included 2 subgroups of disease-free controls: women with symptoms suggestive of endometriosis (pain ± infertility) and women who were symptom-free. The rationale for inclusion of the 2 control subgroups was based on the assumption that pelvic pain may alter the endometrial milieu and consequently the miRNA profile irrespective of endometriosis. Thus, the choice of control subgroups in the studies of endometriosis may have an influence on the results obtained.

Chapter 7 includes a prospective cohort diagnostic accuracy study that implements a stepwise approach for discovery, selection and validation of circulating miRNA biomarkers for diagnosis of endometriosis. Putative plasma miRNA biomarkers were identified and their diagnostic accuracy, alone or in combination with clinical parameters, was assessed.

Finally, the findings of the preceding chapters are summarised in Chapter 8, which also outlines areas for future research. This thesis served as a basis for provisional patent application for a biomarker panel for endometriosis. Since every chapter was written to be read on its own, some repetition and overlap across chapters is inevitable.

1.2 Background to the study

1.2.1 The enigma known as endometriosis

1.2.1.1 Definition of endometriosis

Endometriosis is identified by the presence of endometrial-like tissue in ectopic sites outside the physiologically normal location of the uterine cavity, typically affecting the pelvic organs and peritoneum (1). The term endometriosis is derived from the ancient Greek: *end* means inside, *metra* means womb, *osis* means disease, and was introduced to indicate the presence of ectopic tissue, which possesses the comparable histologic structure and function to the uterine mucosa.

The clinical forms of endometriosis depend on the location and characteristics of ectopic endometrial implants, occurring singly or in combination. Peritoneal endometriosis consists of implants on the surface of the pelvic peritoneum and adjacent organs; ovarian endometriosis is defined by the presence of ovarian cysts lined by endometrioid tissue (endometrioma); and recto-vaginal endometriosis is defined by the presence of a complex mass of endometriotic, adipose and fibro-muscular tissue in the recto-vaginal septum (1). More rare forms of endometriosis include lesions at sites distant from the pelvic cavity, such as in the umbilicus, spleen, liver, pleura, lung, pericardium or brain. Whether different forms of endometriosis are caused by different mechanisms or represent the variants of the same pathologic process remains debatable (2-3). However, endometriotic lesions share common histological features that include presence of endometrial stromal or epithelial cells, chronic bleeding and signs of inflammation.

Since the first description by Carl Von Rokitansky in 1860 (4), significant knowledge has been gained on the epidemiology, genetics and pathological mechanisms of endometriosis. However, the aetiology and pathogenesis of endometriosis remain poorly understood and little progress has been made in relation to its diagnosis or cure.

1.2.1.2 Prevalence estimates of endometriosis

Endometriosis affects women of reproductive age of all ethnic and social groups. The true prevalence of the disease is hard to establish. The prevalence estimates vary significantly depending on diagnostic criteria and populations studied and range from 2-22% in the general female population and in up to 82% in women suffering from either abdominal pain or infertility (5). Figures that are commonly cited in literature by the experts in the field refer to

prevalence of 6-10% in the general population, and 35-50% in women with pain, infertility or both (1, 6-7).

1.2.1.3 Clinical presentations of endometriosis

Endometriosis is a disease with a broad differential diagnosis and a wide spectrum of clinical symptoms from completely asymptomatic to severely debilitating presentations.

Chronic pelvic pain and infertility are the most prominent features of endometriosis, reported in 98% and 69% of affected women, respectively (8). Endometriosis accounts for more than one third of the diagnoses made in women with chronic pelvic pain with significant variability of reported prevalence in this group ranging from 2 to 74% (9). More than two thirds of adolescent girls (69.6%) with chronic pelvic pain and failure to respond to medical treatment were found to have endometriosis at laparoscopy (10). Severe forms of disease result in adhesions and distortion of local anatomy that contribute to clinical presentation. Overall, endometriosis is widely acknowledged as a leading cause of disability in young women (7). The symptoms usually resolve with menopause.

Endometriosis is a leading reason for gynaecologic hospitalisation (11). A population-based, retrospective cross-sectional and longitudinal analysis of 53,385 hospital admissions demonstrated that 27% of women hospitalized for a surgical treatment for endometriosis will be readmitted for additional surgery within 4 years, and one in 10 will have a hysterectomy (12). Another study reported a reoperation rate of 51% within 10 years in women with endometriosis (13). Without treatment, endometriotic lesions tend to progress in severity in about half of the patients (14). A recurrence rate of endometriosis after either medical or surgical treatment is high, estimated as 21.5% at 2 years and 40- 50% at 5 years (15). Taken together, the above observations indicate that although not life threatening, endometriosis is a progressive disease that severely compromises patient health and leads to a repeated necessity for surgical intervention.

1.2.1.4 Endometriosis – systemic disease

There is growing evidence that endometriosis induces systemic effects beyond its common gynaecological presentation. Indeed, women with endometriosis present with a range of health problems. This involves increased risk of certain types of cancer (16-19), auto-immune, endocrine and atopic disorders (20-21), susceptibility to infection (22) and cardiovascular disease (23-24). Consistent with epidemiological observations, endometriosis is associated

with characteristic abnormalities in function of macrophages, B cells, T cells and natural killer (NK) cells along with the presence of auto-antibodies, inflammatory mediators and other active substances (i.e. growth factors, oxidative stress parameters and vasoactive substances) in the systemic circulation, peritoneal fluid and endometrial tissue, reviewed in (1, 6, 25-30).

1.2.1.5 Predisposing risk factors for endometriosis

Risk factors for endometriosis, include vaginal outflow obstruction, longer exposure to menstruation (early menarche, late menopause, short cycles and heavy periods) (31-32). In addition, in-utero exposure to potent oestrogens (diethylstilbestrol), exposure to environmental toxins (chemotoxic organo-chlorines such as dioxin and polychlorinated biphenyl), and alcohol and caffeine intake have been identified as additional risk factors (31-35).

1.2.1.6 Genetics of endometriosis

Animal and human studies have revealed a genetic predisposition to endometriosis with an incidence of up to 6-7 times higher among first-degree relatives of affected subsets than in the general population (36-39). Familial and epidemiologic studies indicate that endometriosis follows a polygenic/multi-factorial inheritance pattern (40). Although multiple genetic variations have been explored by several research groups, earlier replication studies are commonly inconsistent. In the meta-analyses by Guo (2006), no linkage or functional data were found to support a putative relationship of candidate gene polymorphisms with endometriosis (41). A recent genome-wide association meta-analysis of 4,604 endometriosis cases and 9,393 controls of Japanese and European ancestry identified seven genetic variants associated with endometriosis, each of which was replicated in an independent cohort (42). Thus, the search for genetic variants predisposing women to endometriosis continues to generate a lot of interest, even though convincing evidence of a specific genetic defect in endometriosis is still lacking.

1.2.1.7 Social and economic aspects of endometriosis

Endometriosis can severely affect quality of life. Symptoms have a pervasive effect on lifestyle, impair psychological and social functioning, and may interfere with education, employment and sexual relationships (43). According to the US Health Interview Survey, the average annual number of days off work associated with sick leave for women with endometriosis has been estimated at 17.8 (44). Women with endometriosis experience a higher rate of depressive

symptoms, a sense of lack of control and powerlessness, feelings of social isolation and lost chance for motherhood, or concerns that their daughters might develop endometriosis (45-46).

Endometriosis has a significant economic impact being associated with substantial health care costs and costs of productivity loss, attaining 22 billion dollars annually in the US; comparable high cost estimates have been reported from Europe (47-48). The endometriosis-associated costs to society are significantly higher when outpatient investigations and fertility treatments expenses are included. In November 2011, an Australian initiative, endorsed by pain Australia and the faculty of pain medicine, assessed the burden associated with conditions causing chronic pain in the pelvic pain report (49). The estimated direct cost per annum of medical and surgical treatments for endometriosis in Australia is 6 billion dollars for women and 600 million dollars for adolescent girls. This includes the direct cost per annum of medical and surgical treatments and indirect costs of parents taking leave from work and disability pensions. Although the exact estimates regarding the costs of endometriosis are lacking, the literature that endometriosis places a considerable burden on patients and society is indisputable (50). Given its high prevalence, progressive nature and the impact on general health and social wellbeing, endometriosis is recognized as one of the top major public health problems (51).

1.2.1.8 Pathogenesis of endometriosis

1.2.1.8.1 Pathogenetic models

Endometriosis remains a disease of uncertain aetiology. Several mechanisms have been proposed to have a role in the genesis of endometriotic lesions. Sampson's hypothesis suggests implantation and development on the peritoneal surface of refluxed endometrial cells that reach the abdominal cavity after retrograde menstrual flow through the fallopian tubes (52). Induction of retrograde menstruation in primate models has been successfully used to induce endometriosis (53). Sampson's theory is also supported by the facts that endometriosis almost exclusively affects menstruating species and does not appear before menarche (54). Women with endometriosis have higher volumes of retrograde menstrual blood flow and the disease is more common in women with vaginal outlet obstruction and thus a higher extent of retrograde menstruation (55-56). Other theories that propose either local differentiation of the mesothelial peritoneal cells into endometrial tissue (coelomic

metaplasia hypothesis) or transport of endometrial tissue through blood or lymphatic vessels from the uterine cavity to distant sites (vascular theory) may explain certain types of endometriosis, however they are more challenging to prove or refute (57).

Sampson's theory remains the most widely accepted explanation for the development of endometriosis. Current modifications of this concept assume underlying endometrial or immune alterations that are imperative for implantation of endometrial tissue at the ectopic sites and help to explain why amongst the majority of women experiencing retrograde menstruation (90%), only certain individuals (10-15%) develop the disease (1). It is thought that molecular abnormalities of the endometrium or the substrate tissue promote survival of the implants; whereas abnormalities of the immune system contribute to diminished clearance of the foreign tissue from the atypical location (1).

Investigation of the biochemical mechanisms involved in endometriosis clearly indicates that endometriosis represents an oestrogen-dependent, inflammatory systemic pathologic condition, rather than a local disease of the peritoneum. Inflammation, one of the prominent features of the disease, is associated with overproduction of prostaglandins (PGs), cytokines, matrix metalloproteinases (MMPs) and chemokines. Chronic inflammation is responsible for the main clinical features of the disease and mediates pain via stimulation of pelvic nerve endings and presumably also underpins infertility (6). Peritoneal fluid in women with endometriosis, which contains activated macrophages and pro-inflammatory mediators, has been shown to have a deleterious effect on sperm function, embryo quality and uterine endometrial receptivity that would thereby result in reduced pregnancy rate (58-59). Steroid-related pathologic processes involved in endometriosis include excessive production of oestradiol (E2) and development of progesterone (P4) resistance. E2 in turn enhances proliferation of endometriotic tissue and promotes inflammatory response and production of PGs that add to pain and infertility (60). Excessive PGs and inflammatory mediators produced by endometriotic lesions further induce E2 production by positive feedback mechanisms via nuclear oestrogen receptors (ER) and enzymatic pathways (6). An aberrant immune response, inflammation and hormonal aberrations are the most prominent features of the disease that have been observed in 3 major compartments: eutopic intrauterine endometrium, peritoneal fluid and blood.

The cause-effect relationship between inflammation and the progression of endometriosis is not established. The implantation of endometrial tissue at an ectopic site has been shown to

be a complex interaction between the peritoneum and the ectopic endometrium. Infiltrating host site cells such as neutrophils, macrophages, vascular endothelial cells and myofibroblasts participate in cellular events in the ectopic endometrium including tissue breakdown, inflammation, activation of the healing response and glandular remodelling and repair. This implies that endometriotic lesions might induce a systemic response that alters the eutopic endometrium. Alternatively, underlying systemic immune alterations might favour progression of endometriotic lesions and impact eutopic endometrial function.

1.2.1.8.2 Eutopic endometrium

Eutopic endometrium in women with endometriosis is postulated to have endogenous characteristics that predispose to the attachment, invasion, and survival of eutopic endometrium on the peritoneal surface. The eutopic endometrium in women with endometriosis appears histologically normal; however comparison with unaffected women revealed molecular distinctions that include aberrant steroidogenesis, P4 resistance, and over-production of prostaglandins, inflammatory mediators and growth factors (61-63).

The inflammatory characteristics include over-expression of MMP-7 and MMP-11, involved in degradation of cellular matrix components and hepatocyte growth factor (HGF) that stimulates proliferation of endometrial cells. As well, several interleukins (IL-1, IL-6, IL-8), epidermal growth factor (EGF), fibroblast growth factor (FGF), insulin-like growth factor (ILGF), platelet-derived growth factor (PDGF), vascular endothelial growth factor (VEGF) are dysregulated and potentially contribute to angiogenesis and establishment of endometriotic lesions (1, 6, 28, 64). Increased expression of glutathione peroxidase and catalase in eutopic endometrium in endometriosis suggests the possibility of free radical involvement in endometriosis (65).

Overproduction of E2 and P4 resistance is a distinctive feature of endometriosis. Endometriotic tissue and eutopic endometrium in affected women express a complete set of steroidogenesis genes, with aberrant expression of aromatase and nuclear ER [steroidogenetic factor 1 (SF1), ER-β] that lead to continuous local production of E2 (6). Eutopic endometrium in endometriosis demonstrates decreased expression of molecular markers of P4 action (epithelial glycodelin and prolactin) and low P4 receptor levels. Resistance to P4 action leads to decreased P-mediated inactivation of E2 (66). Thereby, increased synthesis and reduced deactivation result in high local concentration of E2 that would act to further promote growth of the endometriotic lesions and enhance inflammation.

Additional findings, associated with decreased receptivity in the endometrium from women with endometriosis, include reduced expression of integrin α and β, two markers of uterine receptivity; leukaemia inhibitory factor (LIF) and HOX genes, required for decidualisation; pathologic upregulation of endometrial-bleeding associated factor in the implantation phase and aberrant expression of genes involved in cellular mitosis, embryonic attachment, and embryo toxicity (67).

The impact of the peritoneal lesions on eutopic endometrium is clearly recapitulated in the animal models of endometriosis (53, 62, 68-69). Dysregulation of proteins involved in invasion, angiogenesis, cell growth, and steroid hormone action have been observed in eutopic endometrium after induction of endometriosis in baboons (68). Furthermore, CYR61 mRNA, which encodes a pro-angiogenic factor that mediates diverse roles in development, cell proliferation, and tumourogenesis, was found to be elevated in the eutopic endometrium of baboons following peritoneal inoculation with menstrual endometrium, which suggests that endometriotic lesions induce a shift in gene expression patterns in the eutopic endometrium (69).

1.2.1.8.3 Peritoneal fluid

Compromised immune surveillance is thought to be one of the crucial factors in the development of endometriosis. There is much evidence that endometriosis is associated with activation of lymphocytes and peritoneal macrophages that consequently mediate the inflammatory reaction associated with endometriosis. Activated immune cells perpetuate elevated production of intraperitoneal growth factors and cytokines in endometriosis. A concomitant decrease in phagocytosis by macrophages and compromised NK cell activity in peritoneal fluid of women with endometriosis is thought to allow shed endometrial cells to escape immune surveillance and become established in the peritoneal cavity (70-72).

Increased levels of acute inflammatory cytokines found in peritoneal fluid from women with endometriosis, such as IL-1β, IL-6 (73), and tumour necrosis factor- α (TNF-α) (74), may enhance the adhesion of shed endometrial tissue fragments onto peritoneal surfaces and participate in growth promotion of established endometriotic lesions (72). Proteolytic MMPs are presumed to further facilitate implantation on the ectopic sites (72). Gene expression profiling of the peritoneum from subjects with and without endometriosis demonstrated up-regulation of MMP-3, intercellular adhesion molecule-1 (ICAM-1), transforming growth factor-β (TGF-β), and IL-6 (75). Monocyte chemoattractant protein-1 (MCP-1), IL-8, interferon-γ (INF-

γ) and regulated upon activation normal T cell expressed and secreted (RANTES) that have been detected in peritoneal fluid in endometriosis (76), induce the recruitment of granulocytes, NK cells and macrophages into the endometriotic lesion. Positive feedback loops would ensure further accumulation of these immune cells, cytokines and chemokines in established lesions.

1.2.1.8.4 Peripheral blood

The peripheral blood reflects immune abnormalities in women with endometriosis that involve cell-mediated and humoural immunity with increased incidence of auto- and anti-endometrial antibodies, along with abnormalities in function and increased counts of B cells and T cells (77). In addition, an increased concentration of pro-inflammatory mediators have been described in blood with many of the same factors elevated as seen in peritoneal fluid, supports the central role of a chronic systemic inflammation in the pathogenesis of the disease (27, 78-79).

1.2.1.8.5 Molecular events associated with endometriosis

The molecular events associated with endometriosis have been extensively investigated through studies in endometriosis lesions and eutopic endometrium. Global gene-expression has revealed candidate genes related to tissue invasion, cell survival and growth and aberrant steroidogenesis (80-93). The most characterized molecular abnormalities, which involve up-regulation of the gene transcripts that mediate the abovementioned responses, are presented in Table 1-1 (94).

Table 1-1 Genes aberrantly expressed in endometrium in association with endometriosis

Up-regulated transcript	Associated function
IL-1, TNF-α	pro-inflammatory response
CREBBP, HIF-1α, VEGF	cell proliferation and angiogenesis
IRS-1 , KRAS	cell proliferation and survival
CDKN1A/1B	cell cycle repressors (mediate survival of ectopic lesions)
COL1A1, COL3A1, COL5A1	tissue remodelling
BCL-2	apoptosis resistance
COX-2	proliferation and steroidogenesis

CREBBP - CREB binding protein; HIF- hypoxia inducible transcription factor; IRS - insulin receptor substrate; KRAS - Kirsten ras oncogene homolog; CDKN - cyclin-dependent kinase inhibitor; COL - collagen-matrix proteins; BCL - B-cell lymphoma protein; COX – cyclooxygenase.

The most significant signalling pathways associated with endometriosis include TGF-B signalling that promotes tissue remodelling and invasiveness, inhibitor of kappa light polypeptide gene enhancer in B-cells, kinase beta (IKBKB)/ nuclear factor kappa B (NFkB) pathway involved in inflammation, mitogen-activated protein kinase (MAPK) signalling involved in proliferation, as well as JUN/FOS signalling involved in cell migration and cancer metastasis (94).

Interestingly, the eutopic endometrium in women with endometriosis shares molecular events observed within the lesions, which supports the assumption that endogenous abnormalities in endometrium might predispose to the attachment, invasion, and survival of eutopic endometrium on the peritoneal surface. Global gene-expression profiling of endometrium from women with endometriosis has revealed candidate genes related to tissue invasion, cell survival, angiogenesis, impaired endometrial receptivity and decreased apoptosis as compared with endometrium from disease-free women (95). Additionally, a parallel gene expression analysis of endometrial specimens obtained during the early secretory phase demonstrated a signature of P-regulated genes, confirming the observation of an attenuated P4 response (96).

1.2.1.9 Diagnosis of endometriosis

1.2.1.9.1 Diagnostic delay in endometriosis

Endometriosis remains a significantly under-diagnosed disorder. Women with endometriosis usually experience substantial diagnostic delay, frequently associated with a long and expensive diagnostic search. Studies reported in different health care systems reveal a mean delay in diagnosis of 4 to 12 years, which is longer in younger women (97-101). Two thirds of women with surgically diagnosed endometriosis reported onset of symptoms during adolescence with significantly longer time to diagnosis than those whose symptoms began as adults, occurring due to the combination of a longer period before seeking medical attention and a longer time to receiving a diagnosis (8).

The delayed diagnosis is mainly due to variability in presentation, lack of pathognomonic features and deferred referral to secondary care. Diagnostic laparoscopy is the standard investigation of endometriosis and is considered the only diagnostic test that can reliably rule out the disease. The absence of an accurate non-invasive diagnostic test and reluctance for surgery significantly contribute to diagnostic delay.

1.2.1.9.2 Does the delay in diagnosis matter?

Increased time to diagnosis has been correlated with more severe forms of the disease (102). This may result in more severe clinical presentations and poorer treatment outcomes, which could have been avoided with earlier diagnosis. Advanced endometriosis is associated with higher incidence of infertility, a fall in pregnancy rates obtained, and lower success of ART cycles involving poor ovarian reserve, and low oocyte and embryo quality (58, 103-106).

Accurate diagnosis allows early referral to secondary care, tailoring of the most appropriate therapy and multidisciplinary follow up for associated disorders. Early diagnosis potentially limits the number of investigations, thus minimizing cost and possible associated side effects of management (107). Moreover, prompt diagnosis diminishes associated psychological and social disadvantages. It provides relief from fear of existence of other significant pathologies, gives women a reasonable explanation for their symptoms, legitimizes absence from work and allows referral to support groups (98, 108).

1.2.1.9.3 The "gold standard" method for diagnosis of endometriosis

The diagnosis of endometriosis is mainly a histological one, achieved after surgical excision of the suspected lesion, commonly performed by laparoscopy. The classic endometriotic lesions lie under the peritoneal surface and are typically of blue-black colour. The endometriotic lesions can appear in atypical forms and may exhibit red, brown, black, white or yellow colours (109). Histological confirmation of diagnosis is strongly recommended by most authorities, especially in the case of atypical lesions (110). During laparoscopy endometriosis is classified according to the revised American Society of Reproductive Medicine criteria (rASRM) that include location and size of the lesions (111). This classification allows uniform documentation of the extent of disease but has little value in clinical implications due to lack of correlation between laparoscopic staging and the severity of disease (112-114).

There are several reasons to review the place of laparoscopy in the diagnosis of endometriosis, including high costs, invasiveness and arguable diagnostic accuracy. Laparoscopy requires trained personnel, together with full operating theatre and recovery facilities. It carries the risk of complications that, although rare, can lead to substantial morbidity and even mortality, including 0.1/1000 risk of death, 2.4% of bowel, bladder or blood vessel injury, in which the majority of cases will need extensive surgical repair (115-116). Laparoscopy can lead to anaesthesia-related complications and result in increased adhesion formation that further compromise fertility. Only one third of diagnostic laparoscopies in women with suspected

disease reveal endometriosis, thus two thirds of women undergoing surgery are exposed to unnecessary risks (117).

It is increasingly apparent that laparoscopy fails to diagnose endometriosis with the accuracy expected from a "gold standard" test. The quality of diagnosis depends on the skills of the surgeon to visualize suspected lesions and on the ability of pathologists to recognize the features of endometriosis within the excised tissue, both of which are subject to considerable inter-individual variability (118). The positive predictive value of laparoscopic visualization of endometriosis is estimated to be about 43-45% and depends on lesion type and location of lesion (119-120). A systematic review that assessed the diagnostic value of laparoscopy in endometriosis reported the overall sensitivity of 94% (95% CI 80–98%) and specificity of 79% (95% CI 67–87%) according to the four studies included in the meta-analysis. However the authors highlighted the paucity of good quality literature available with need for cautious interpretation of the results (121). The main reasons for poor visual diagnosis include confusion with other types of lesions and the presence of microscopic endometriosis in visually normal peritoneum (122). Positive histology for endometriosis confirms about 75% of excised lesions and 58% of subtle lesions (123-124). Up to 59% of histologically documented endometriosis was not reported by the surgeon at laparoscopy (125). Histological confirmation increases diagnostic accuracy with an overall positive predictive value of 86.5% when biopsies are obtained from multiple sites (126). Unfortunately, while remaining the best diagnostic option available, laparoscopy verified by histology fails to confirm the disease in a subset of women (127). Advances of laparoscopic techniques with implementation of auto-fluorescence, narrowband imaging, and use of the endorectal probes promise an advantage to conventional laparoscopic modalities in detecting endometriotic lesions (128-130).

Considerable costs, associated complications, and unsatisfactory accuracy, particularly in the diagnosis of mild and atypical forms of disease, underpin the need for a reliable non-invasive diagnostic test for this debilitating progressive disease. Provided surgery effectively alleviates symptoms and improves fertility, a significant proportion of patients will require laparoscopy despite the initial method of diagnosis. In this case, non-invasive diagnostic tests would allow a more specific focus on the subgroup of women that will benefit from the intervention.

1.2.1.9.4 Non-invasive approaches for diagnosis of endometriosis

The need for a non-invasive test for endometriosis has been acknowledged worldwide (131). The abundance of recently published literature concerning the role of clinical, imaging and

laboratory markers or their combinations in the diagnosis of endometriosis shows variation in accuracy estimates and in the populations studied. Currently, none of the non-invasive tests is particularly helpful in making or confirming a diagnosis of endometriosis (132-133). The comparative appraisal of the existent diagnostic approaches to endometriosis is summarised in Table 1-2.

Table 1-2 Comparative appraisal of current diagnostic approaches to endometriosis

Test	Cost	Invasiveness	Specificity	Sensitivity	Safety
Laparoscopy	High	High	High	Moderate to High	Potential risk
Questionnaires	Low	Low	Low	Low	Safe
Ultrasound	Moderate	Moderate	High	Low to Moderate*	Safe
MRI Imaging	High	Moderate	High	Low to Moderate*	Safe
Serum markers	Low	Low	Some High	Low to Moderate*	Safe
Endometrial markers	Moderate	Moderate	Variable# (potentially high)	Variable# (potentially high)	Safe

*Increased in severe disease or presence of endometrioma;
Dependent on immunohistochemical stain and research group.

1.2.1.9.5 Clinical parameters

The decision about progressing to an invasive procedure is usually based on a patient history, physical examination and imaging. Although certain symptoms are more frequently associated with endometriosis, history alone is a poor discriminator given the broad differential diagnosis and the heterogeneity of presentations (134). Self-administered questionnaires based on symptoms, demographic characteristics and medical history have proven to be efficient in highly symptomatic women, mainly for better location of endometriotic sites and planning of surgery (135-136). Given the high prevalence of asymptomatic endometriosis of 2-50% in various reports, questionnaires are of no value as an independent diagnostic tool in the wider cohort of patients (137). Abnormalities on clinical examination, including nodules and indurations on retro-cervical and utero-sacral ligaments, decreased uterine mobility and adnexal mass correlate with findings of endometriosis on laparoscopy in 70-90% (138-139). However the differential diagnosis of these clinical findings is broad. Abnormal findings on clinical examination can raise clinical suspicion and be of value for further investigations but are not sufficient for an accurate diagnostic test. A normal pelvic examination does not exclude endometriosis as most women, particularly those with mild or moderate disease, show no abnormalities on physical examination. Laparoscopically proven disease has been diagnosed in more than 50% of women with normal pelvic examination prior to surgery (139).

The utility of traditional clinical evaluation methods in patients with suspected endometriosis is limited, especially in women with mild or moderate disease.

1.2.1.9.5.1 Imaging modalities

Widely implemented in gynaecological practice, ultrasound (US) correctly diagnoses ovarian masses but is unreliable in diagnosis of non-ovarian endometriosis (140-141). Moore et al. systematically reviewed 67 papers on the validity of US for the detection of pelvic endometriosis, out of which seven fulfilled the inclusion criteria and focused on US imaging of ovarian endometriomas. Sensitivities, specificities, positive likelihood ratio (LR+) and negative likelihood ratio (LR−) in six studies using grey-scale ultrasonography ranged between 64-89%, 89-100%, 7.6-29.8 and 0.1-0.4, respectively (141).

Sensitivities of plain transvaginal ultrasonography for diagnosis of non-ovarian pelvic endometriosis have been reported to range from 34% to 64% and specificity from 50% to 88% (139, 142), in which higher accuracy estimates have been reported for deep infiltrating lesions (143). Studies of modified US scanning (tenderness-guided approach, adding of water contrast in rectum, rectal US) or using bowel preparation suggest good predictive values for diagnosis of pelvic, rectal and vaginal involvement (144-148). For example, a study on 72 patients with different locations of endometriosis demonstrated sensitivity of 74-91% (95% CI, 79–97%), specificity of 88-89% (95% CI, 81–93%) for the diagnosis of endometriosis in vaginal wall and rectovaginal septum when associated with a tenderness-guided transvaginal sonography. The authors reported lower sensitivity (ranging 67-33%) with a comparable specificity for other locations of endometriosis (144). Notably, most of these studies enrolled a highly selected group of symptomatic patients and specially trained personnel. Thus it is difficult to extrapolate this data to the general population with endometriosis. Magnetic resonance imaging (MRI) appears to display good diagnostic sensitivity and specificity in all localizations of endometriosis, particularly in deeply invasive lesions (149-150), but the accuracy estimates of MRI diagnosis vary between the studies and largely depend on the size and depth of invasion of the lesions, reviewed in (151). The diagnostic ability of both US and MRI is highly dependent on the operators' skills. Negative results of imaging cannot be used as definitive evidence to exclude endometriosis and it is still unclear if any of the imaging techniques are a reliable diagnostic option in women with mild or superficial forms of endometriosis in the absence of gross anatomical findings.

1.2.1.9.5.2 Laboratory tests

Recent endometriosis research, including advances in genomic, proteomic and microarray technologies, has focused on the development of accurate, non-invasive diagnostic methods. Reviewing the literature, we found more than 1000 papers exploring laboratory based diagnostic tests for endometriosis. Multiple biomarkers in peripheral blood, endometrium or peritoneal fluid have been evaluated as candidate diagnostic tools. Endometrial fluid aspirated from the uterine cavity has been suggested as a potential sample for non-invasive diagnostics (152). Factors associated with abnormal steroidogenesis, inflammation, and altered cellular and humoural immunity have been correlated with the presence of the disease. Investigated markers for the diagnosis of endometriosis are presented in Table 1-3.

Table 1-3 Serum and endometrial markers investigated for diagnosis of endometriosis

Serum markers	
Growth factors	TGF-α, TGF-β, EGFR, VEGF, SF, HGF, FGF, PDGF, EGF, IGF-I
Hormones	LH, P, Leptin
Proteolytic enzymes	MMP-1, MMP-2, MMP-3, MMP-9, TIMP-1, TIMP-2
Glycoproteins	Ca-125, Ca-19-9, Follistatin, Osteopontin, PP 14
Soluble adhesion molecules	sICAM, sVCAM-1, sEcadherin, sEselectin
Immune cells alterations	T helper/T suppressor ratio, NK activity, total complement levels
Autoantibodies	anti-endometrial, anti-phospholipid, anti-sialyate-T, anti-Thomsen Friedenreich antigen, anti-transferrin, anti-LDL, HLA class 1/ class 2, anti-laminin-1, anti-2HSG, anti-thyroid
Cytokines	IL-1, IL-6, IL-8, IL-12, IL-13, MCP-I, MIF, Fas ligand, TNF-α
Endometrial markers	
Cell adhesion molecules	Integrins, Selectins, Cadherins
Hormones/hormone receptors	Aromatase P450, ER, PR
Proteolytic enzymes	MMP-2, MMP-3, TIMP-2, TIMP-3, MTMMP-1
Other polypeptides	PP 14, Osteopontin, PGP 9.5, ANXA 1, CYR61

ANXA – annexin; anti-2HSG - 2 Heremans-Schmidt glycoprotein; Ca-125 - Cancer antigen 125; CYR61 - cysteine-rich, angiogenic inducer 61; EGFR - epidermal growth factor receptor; HLA - human leukocyte antigen; IGF – insulin-like growth factor; LDL - low density lipoprotein; LH –luteinising hormone;

MIF - migration inhibitory factor; MTMMP - membrane type matrix metalloproteinase; TIMP - tissue inhibitors of MMP; PGP 9.5 - protein gene product 9.5; PP 14 - serum placental protein/ glycodelin A; PR – progesterone receptor; sEcadherin - soluble E-cadherin; sEselectin - soluble E-selectin; sICAM - soluble intercellular adhesion molecules; sVCAM - soluble vascular cell adhesion molecule.

The literature varies in accuracy estimates and the populations studied. Most of the studied markers, either single or combined in a panel, appear to have low accuracy and to lack of sufficient diagnostic power (152-157). Although many of the reported markers distinguish women with and without endometriosis in small pilot studies, there are profound differences between research groups in reported sensitivities and specificities for most of the tests. There

is little consensus in the published literature and most studies are of low methodological quality.

Ca-125, the glycoprotein expressed by coelomic epithelium and the most extensively studied marker, has a limited value as a screening or a diagnostic test for endometriosis, especially in mild-moderate forms of the disease (158). The meta-analysis that assessed the diagnostic accuracy of Ca-125 in endometriosis showed that Ca-125 has a limited diagnostic performance. Depending on the chosen cut-off values, Ca-125 has been reported to show specificity of 90% with sensitivity of 28% or specificity of 72% with sensitivity of 50%. Another promising biomarker for endometriosis is the immunohistochemical identification of small nerve fibres in the functional layer of the endometrium using an antibody against PGP 9.5 (159). A further study demonstrated that the density of small nerve fibres was about 14 times higher in endometrium from patients with minimal–mild endometriosis compared to disease-free women. The authors demonstrated that the combined analysis of neural markers such as PGP 9.5, anti-vasoactive intestinal peptide (VIP), anti-neurofilament protein and anti-substance P could predict the presence of minimal–mild endometriosis with 95% sensitivity, 100% specificity and 97.5% accuracy (160). These findings require validation in large prospective clinical trials.

Given the association of endometriosis with various molecular events, many research groups anticipated that messenger RNA (mRNA) and proteomic assessments would reveal diagnostic markers. However many transcripts thought to be associated with endometriosis were not identified in the published microarray studies of endometriosis and *in silico* analysis revealed a mismatch between the expression levels of transcriptomes and proteins associated with the disease (161-163).

1.2.1.10 Summary

Decades of research suggest that endometriosis is a complex disorder, with varying severity, onset and progression. However, endometriosis remains an enigmatic under-diagnosed disease with substantial associated morbidities. No useful specific diagnostic biomarker for endometriosis has been identified. Understanding the mechanism of disease and establishing a reliable non-invasive test remain major priorities in endometriosis research, which is currently focused on new genomic approaches.

1.2.2 MiRNAs – small molecules with a huge potential

1.2.2.1 MiRNAs as regulators of gene expression

MiRNAs comprise a family of highly conserved 9-22 nucleotide long non-protein-encoding RNAs that post-transcriptionally regulate gene expression via suppression of specific target mRNAs (164-165). MiRNAs are involved in the regulation of translation and degradation of target mRNAs through base pairing to complementary or partially complementary sites in the 3'-untranslated regions (3'-UTR) of mRNAs. As regulators of post-transcriptional gene expression, miRNAs play an essential role in a large number of biological and pathological processes, including the regulation of development, cell proliferation, apoptosis, and differentiation.

MiRNAs represent only a small fraction (~0.01%) of the total RNA mass. However, since their initial discovery in Caenorhabditis elegans (C. Elegans) approximately two decades ago, miRNAs have been found to regulate approximately 30% of the human genome and are involved in multiple biologic pathways, such as cell survival, proliferation and metabolism (166-169). Currently, 25141 mature miRNA sequences have been identified in 193 species, as listed on the latest version (v.19) of miRBase sequence database, including 2216 mature human sequences (170). The post-transcriptional gene repression by miRNAs involves complex systems of interactions in which each miRNA can target several mRNAs and each mRNA can be the target of different miRNAs. Consequently, each miRNA can regulate, on average, the expression of approximately several hundred target genes (166). Some miRNAs with common mRNA targets are co-expressed from a single miRNA gene cluster, generating an amplified repressive effect on cellular functions. Furthermore, miRNAs and their mRNA targets can form regulatory loops, through which reciprocal regulation provides additional control of gene expression (165-166). It is emerging that miRNAs act via complex networks that integrate thousands of interacting miRNA-target mRNA associations and thus fine tune target protein expression.

1.2.2.2 MiRNAs – biogenesis and function

MiRNAs originate from large precursors and their biogenesis is governed by many regulatory checkpoints. The majority of mammalian miRNA genes are embedded in intronic regions of protein encoding genes and are transcribed in the nucleus into precursor molecules called primary miRNA (pri-miRNA) (171). Pri-miRNAs fold into hairpin structures containing

imperfectly base-paired stems and are processed into 60- to 100-nt hairpins known as precursor miRNAs (pre-miRNAs) by the enzyme Drosha in the cell nucleus (172). The pre-miRNAs are exported from the nucleus to the cytoplasm by exportin 5 (173), where, in general, they are cleaved by the endonuclease Dicer to yield imperfect double stranded miRNA (174). The current belief is that the "guide strand", known as the miRNA, binds to RNA-induced silencing complex (RISC) where it produces its effect on target gene translation. In contrast, the "complementary strand", known as miRNA* (miRNA star), is degraded and was initially thought to have no functional relevance. However recent reports have noted that miRNA* is readily detectable and can play a significant role in gene regulation (167). The conserved seed sequence of the miRNA provides target specificity by base-pairing to target mRNAs, whereas Argonaute proteins (Ago1-4) and other RISC ribonucleoproteins execute the various forms of miRNA-directed RNA processing (175). Mammalian miRNAs bind by imperfect base-pairing to multiple sites in 3'-UTR and sometimes protein-encoding open reading frames of target mRNAs. Partial complementarity generally leads to mRNA decay and/or translational inhibition, whereas highly complementary target sites (which are mainly confined to plants), result in endonucleotic mRNA cleavage and degradation (176). Additional factors influencing miRNA target repression include the number of miRNA target sites within an mRNA sequence, as the magnitude of miRNA directed translational repression is amplified by target site multiplicity. The repressed mRNAs accumulate in cytoplasmic structures called P-bodies where they are degraded or stored (175). During cellular stress, miRNA repression appears to be reversible; there is evidence that mRNAs can be released from P-bodies and become transactionally active (177). There is also evidence that miRNAs might have a stimulatory effect on target mRNA translation, but this appears to be less common (178).

1.2.2.3 MiRNAs in human disease

As miRNAs influence most of the biological processes, miRNA-based regulation has a major role in the aetiology and pathogenesis of a variety of disease states (164). There is a constantly increasing amount of data connecting aberrant miRNA expression to the development of various diseases, including malignancies, heart disease, hypertension, diabetes, pregnancy, immune and reproductive diseases and others (94, 167-169, 179-197). MiRNA mutation have been shown to have a pathogenic role in human genetic diseases (189). MiRNA research has opened new horizons in our understanding of molecular pathogenetic events of the disease and has brought the development of the diagnostic markers to a new level.

1.2.2.4 MiRNAs as biomarkers

MiRNAs have been detected in most tissues and biological fluids. Unlike other RNAs, miRNAs are not subject to degradation by RNases and therefore are more stable than mRNAs and can be reliably extracted from archived frozen and formalin-fixed paraffin embedded tissues (198). MiRNA profiling appears to be more accurate than mRNA profiling in disease classification (188, 199) and therefore are thought to have considerable potential as biomarkers for targeted screening of many diseases. The discovery of miRNAs in the systemic circulation in a highly stable cell-free form highlighted the possibilities for development of blood miRNA-based diagnostics (200-203). While the potential diagnostic usefulness of circulating miRNAs has been observed in a range of diseases (200-201, 203-204), studies on miRNA in endometriosis are just beginning to emerge.

1.2.2.5 MiRNAs in endometriosis

The studies on miRNAs in endometriosis mainly addressed miRNA profile in endometrial tissue. Five previously published large-scale experiments that explored the miRNA profile in eutopic and/or ectopic endometrial tissue in association with endometriosis used either deep sequencing (205) or hybridisation microarray platforms (206-209) and indicated panels of the differentially expressed miRNAs (Table 1-4). These studies compared different types of tissue [endometrioma vs. eutopic endometrium in endometriosis (207), peritoneal lesions vs. eutopic endometrium in endometriosis (208), endometrioma vs. eutopic endometrium in controls, eutopic endometrium in endometriosis vs. in controls (206), or multiple comparisons of peritoneal implants vs. eutopic endometrium in endometriosis and in controls (209)], showing only partial concordance between the obtained results (Figure 1.1).

Table 1-4 Global miRNA profiling studies in endometriosis

Study	Sample tested	Cycle phase	Results
Burney RO, 2009	eutopic in endometriosis (n = 4) vs. eutopic controls (n = 3)	Early secretory	6 miRNAs
Ohlsson Teague EM, 2009	eutopic vs. ectopic (n = 8) in endometriosis	Proliferative and Secretory	22 miRNAs
Filigheddu N.J, 2010	eutopic vs. ectopic (n = 13) in endometriosis	Early proliferative	50 miRNAs
Hawkins SM, 2011	eutopic in controls (n = 11) vs. ectopic in endometriosis (n = 10)	Proliferative and Secretory	22 miRNAs
Pan Q, 2007	paired ectopic-eutopic in patients (n = 4), ectopic in pts (n = 4), eutopic in controls (n = 4)	Early-mid secretory	50 miRNAs

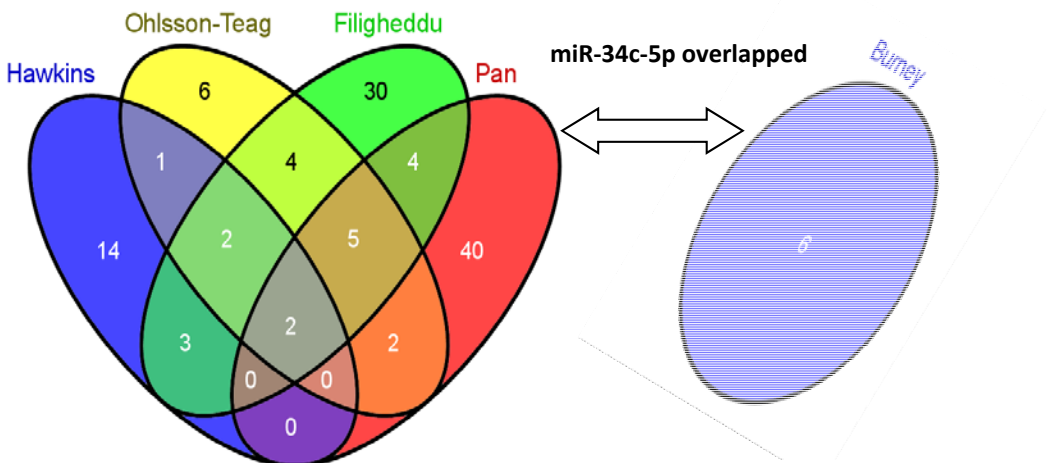


Figure 1.1 Concordance between the published miRNA profiling studies in endometriosis. Venn diagram represents comparison between the miRNA studies and demonstrates the number of overlapping miRNAs.

Recently, several studies addressed the functional relevance of selected miRNAs in endometriosis via characterization of miRNA-mRNA relationships and loss of function manipulations in endometrial and ectopic endometrial cell lineages (Table 1-5).

Table 1-5 Functional studies of the selected miRNAs in endometriosis

Study	miRNA tested	Sample tested	Conclusion
Dai L, 2012	miR-199a	Endometrioma vs. eutopic endometrium (pts, n = 12; ctrl, n = 12)	Increase in cell invasion, activation of NFkB-pathway, elevation of IL-8
Lin SC, 2012	miR-20a	Ectopic vs. eutopic endometrium (pts, n = 37; ctrl, n = 17)	Up-regulation of angiogenic genes and FGF-9, stimulation of proliferation
Petracco R, 2011	miR-135a/b	Eutopic endometrium (pts, n = 32; ctrl, n = 50)	Down-regulation of HOXA-10 that regulates endometrial receptivity
Ramon LA, 2011	miR-125a, miR-222 miR-17-5p	Endometrioma vs. eutopic endometrium (pts, n = 58; ctrl, n = 38)	Lower expression of VEGF-A; higher expression of the angiogenic inhibitor TSP-1

Red colour – up-regulated miRNAs in endometriosis; green colour – down-regulated miRNAs in endometriosis.

Functional *in silico* analyses have identified molecular networks regulated by miRNA transcripts and linked miRNAs with the establishment of endometriotic lesions (Figure 1.2), as comprehensively presented in (94, 210). However, the evidence supporting the hypotheses regarding the mode of action of miRNAs in endometriotic disease is largely indirect and is predominantly based on computational analyses.

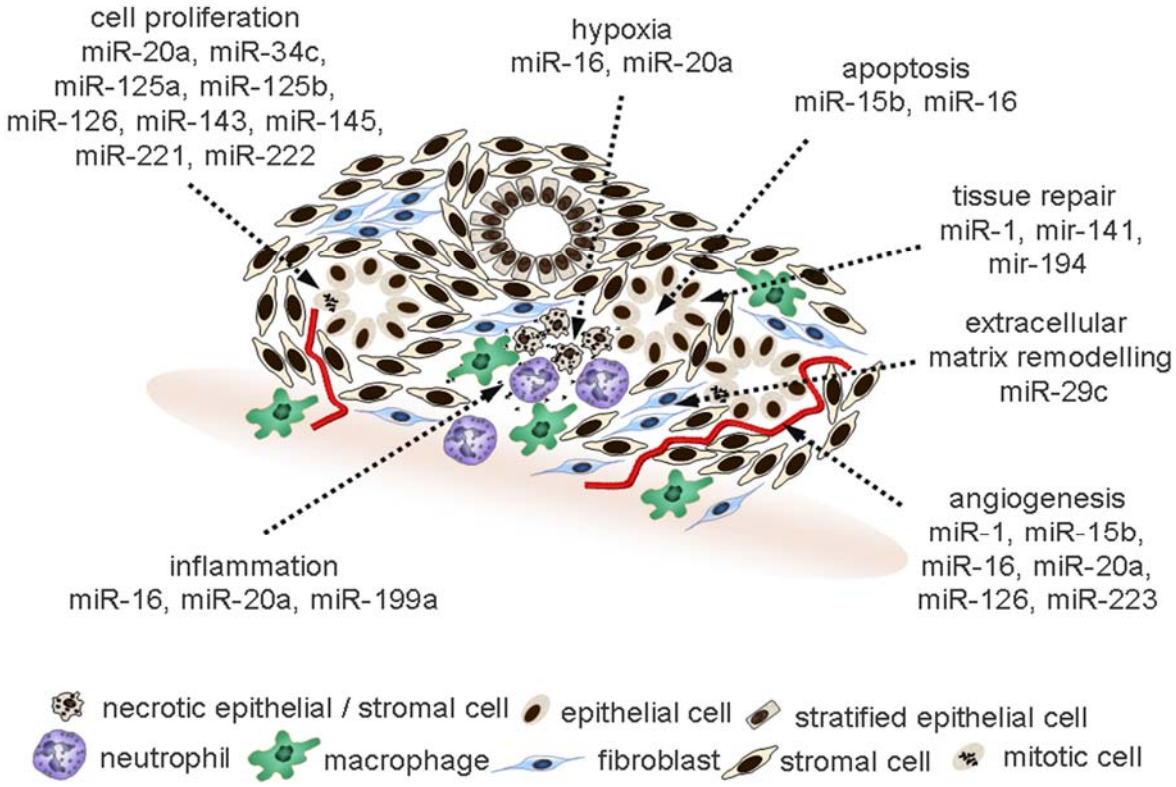


Figure 1.2 Molecular events associated with the establishment of endometriotic lesions and the list of the associated miRNAs. Adopted from Ohlsson Teague EMC et al., 2010 (94).

Two studies exploring circulating miRNAs in endometriosis have been published (211-212), when this thesis was in the final stages of drafting. The authors identified largely independent panels of endometriosis-associated miRNAs in serum and plasma, focusing on their diagnostic potential. These studies are further discussed in Chapter 7.

1.2.2.6 Circulating miRNAs – origins and putative mechanism of action

It is presently unclear which molecular mechanisms are responsible for alterations in serum miRNA levels in certain disease conditions. Earlier observations that circulating miRNAs reflect passive release from the damaged cells (213) have been recently challenged by the observation of actively secreted miRNAs from tissues or cells, which raises the prospect that miRNAs might serve as messengers to influence gene transcription in distant target sites (214-215). Growing evidence suggests that circulating miRNAs can reside in microvesicles and lipoprotein complexes, including exosomes, microparticles, and apoptotic bodies, which offer protection to circulating miRNAs from endogenous RNase activity (215-219). Exosomes and

microparticles are formed through the inward budding of endosomal or plasma membrane, giving rise to intracellular multivesicular bodies (MVB) that contain miRNAs and are later released to the circulation via fusion with the cell membrane (216, 218, 220). The formation and release of exosomes and microparticles is dependent on calcium influx, cytoskeleton reorganization and sphingomyelinase 2 activity (nSMase2), a rate limiting enzyme in ceramide biosynthesis, which is involved in control of exosome release from the cells (220). Whereas the expression of certain miRNAs is higher in microvesicles than in the cells of origin (215), nearly 30% of the released miRNAs *in vitro* and *in vivo* do not reflect the expression profile found in donor cells (221), suggesting that specific miRNAs are selected to be intracellularly retained or released by exosomes. Moreover, different stimuli alter the number, composition and origin of microparticles (222), and modify the release of miRNAs (223), supporting the assumption that packaging and extracellular transport of miRNAs is a highly regulated process. Additionally, the extravesicular mechanisms of transport of circulating miRNAs occurs in association with lipoprotein and protein complexes, including high-density lipoprotein (HDL) (214), Ago2, which is part of the miRNA silencing complex (224-225) and nucleophosmin (NPM1) (218). Recent studies demonstrated that potentially 90% of the plasma and serum miRNAs are co fractionated with the protein and lipoprotein complexes (200). The suggested mechanism of biogenesis and function of circulating miRNAs is presented in Figure 1.3.

Despite the fact that conclusive data on the functional role of endogenous extracellular miRNAs are still missing, increasing evidence suggests that circulating miRNAs can serve as messengers to influence gene transcription and modulate cell function at distant target sites. Exosomal miRNAs have been demonstrated to promote gene silencing similar to cellular miRNAs (220, 226). Further, microvesicles isolated from the circulation of atherosclerosis patients, were noted to induce responses in endothelial cells similar to the response observed in monocytes in atherosclerosis, suggesting their role in mediation of miRNA crosstalk between the donor and target cells (223). Circulating miRNAs are taken up by the recipient cells by endocytosis or through receptor-ligand interactions via binding to receptors present at the recipient cellular membrane capable of recognizing RNA-binding proteins. Similarly, miRNA-lipoprotein complexes have been demonstrated not just to carry circulating miRNAs, but also to affect the expression of target genes with the potential to induce cellular responses.

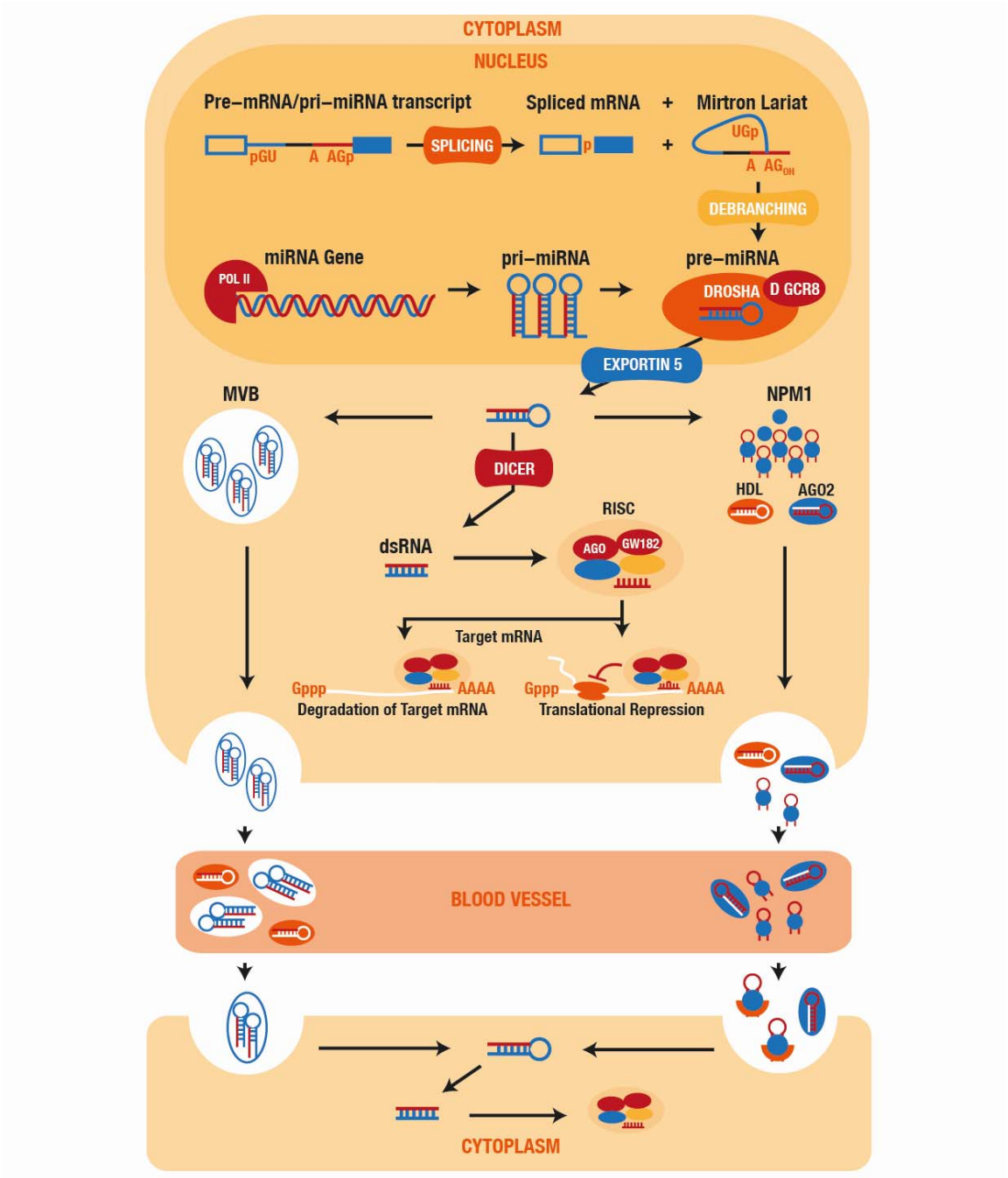


Figure 1.3 Biogenesis, mechanism of action and extracellular secretion of miRNAs. Pri-miRNAs are transcribed by polymerase II (POL II) in the nucleus and processed by Drosha into pre-miRNA. An alternative non canonical pathway is generated by certain debranched introns, called "mirtrons", which undergo splicing and mimic the structural features of pre-miRNAs, entering the miRNA-processing pathway without Drosha-mediated cleavage. Exportin transports pre-miRNA molecules to the cytoplasm where Dicer generates miRNA-miRNA* duplexes. These are converted into single-strand mature miRNA and incorporated into the RISC complex which sequence specifically binds to miRNA target sites on mRNA transcripts, effecting mRNA cleavage and degradation or, if the alignment if imperfect, repression of gene translation. Pre-miRNAs are exported from cells via 2 mechanisms: 1. In multivesicular bodies (MVBs) which release miRNAs into the circulation via fusion with the cell membrane; 2. In association with RNA-binding proteins such as nucleophosmin 1(NPM1), Argonaute2 (Ago2), or high-density lipoprotein (HDL). Circulating miRNAs are taken up by the recipient cells either by endocytosis or, if protein bound, by receptor mediated interactions at the cell surface. MiRNAs internalized by recipient cells can inhibit the expression of target protein-coding genes.

For example, miRNAs within HDL were demonstrated to alter the cellular miRNA pool and resulted in loss of corresponding miRNA targets through activation of a ceramide signalling pathway (214). Release of miRNAs from the donor cells and their uptake by recipient cells represents an additional largely undiscovered level of regulation of circulating miRNAs. More studies are necessary to elucidate how miRNAs are loaded into exosomes and how they can be internalized by recipient cells. Given that endometriosis is acknowledged as chronic-progressive systemic disease and in view of the extensive regulatory capacity of miRNAs, it is tempting to assume that miRNAs have a regulatory role in this condition via the systemic circulation.

1.2.2.7 MiRNAs and endometrial function

Endometrium is a complex, dynamic, steroid hormone-responsive tissue that undergoes highly organized cyclic structural changes in preparation for embryo implantation during the reproductive years. Large number of miRNAs has been identified in reproductive tract tissues, including endometrium in both normal and disease states. *In vitro* and animal studies showed the role of miRNAs in implantation and decidualisation of endometrium, which defined as differentiation of stromal cells in preparation for pregnancy. For example *in vitro* cultures of primary human endometrial stromal fibroblasts (HESF) identified 49 differentially expressed miRNA unique for decidualisation (227). The loss of function studies revealed that miR-222 and miR-29c affected endogenous gene expression of genes important in biological adhesion and extracellular matrix formation (227). An over expression of miR-29c in HESF cells resulted in a blunted decidualisation response (205). Work in the rat and mouse uterus has shown that let-7a and miR-320 are up-regulated in implantation sites (228-229), miR-101a and miR-199a regulate COX2 expression during implantation (230), and miR-21 plays an important role in blood vessel remodelling in implantation through signals from the blastocyst (231). Additionally, a next-generation sequencing study of delayed implantation in the mouse indicated that implantation sites may have additional editing of miRNAs (232). Using *in vitro* cultures of human primary endometrial stromal or epithelial cells, variations in miRNA expression were observed between the different cell types (209, 233), suggesting that miRNAs are involved in regulation of endometrial function and likely to mediate a cross talk between the uterine epithelium and stroma.

1.2.2.8 MiRNAs and ovarian steroids

MiRNAs are known to target genes involved in steroidogenesis, and their expression is modulated by ovarian steroids in in-vitro experiments. MiRNAs have been profiled in the mouse uterus in response to E2 or ER antagonist treatment (234). They have also been profiled using human endometrial epithelial cells from late proliferative phase for the maximum E2 effect and mid-secretory phase for maximum P4 effect of the human menstrual cycle to define the miRNA that may be E- and P-responsive in the endometrium (235). E2 and P4, alone or in combination with their antagonists were shown to alter the expression of miR-20a, miR-21 and miR-26a in isolated endometrial cells (209). The same group demonstrated ovarian steroid dependence of miR-17-5p, miR-23a, miR-23b and miR-542-3p and of their targets, namely cytochrome P450, family 19, subfamily A, polypeptide (CYP19A1), steroidogenic acute regulatory protein (StAR), and COX-2, that play a central role in endometrial proliferation (233). Additionally, regulatory interactions between the ovarian steroids and miRNAs were observed in other steroid sensitive cells or steroid-dependent conditions reviewed in (180, 197, 236-237). The understanding of the interaction between miRNA and ovarian steroids is just beginning to emerge, and E-dependent association with Drosha complex or inhibition of miRNA maturation at the level of processing of pri-miRNAs are among the suggested mechanisms (237-239).

Whereas the enriched supra-physiological hormonal milieu present under experimental conditions can clearly alter miRNA expression, there is no strong evidence that physiological hormonal levels alter miRNA signatures *in vivo*. Although miRNAs appear to be influenced by the menstrual cycle in human endometrium (235, 240-241), there is no data regarding cycle stage effect on circulating miRNAs.

1.2.2.9 Summary

MiRNAs are emerging as important modulators of gene expression and regulators of vital cell processes. A growing body of evidence links miRNAs with a range of pathologic conditions, suggesting their role in the pathogenesis and diagnosis of disease. Evidence of dysregulated miRNA expression in endometriosis tissue raises the prospect of circulating miRNAs as modulators of the systemic effects associated with endometriosis, as well as potential diagnostic tools for the disease.

1.3 The need for the study

Many women with endometriosis suffer a diagnostic delay, which is associated with significant physical and psychosocial morbidity and poses a substantial burden on health service worldwide. The diagnosis of endometriosis requires surgery, which is associated with potentially dangerous side effects, high costs and is one of the reasons for diagnostic delay.

An accurate non-invasive test for endometriosis would reduce the need for a surgical diagnosis, would be more accessible to women, and is likely to lead to an earlier diagnosis and treatment of endometriosis. Although multiple markers have been explored as diagnostic tests for endometriosis, none of them can reliably diagnose endometriosis and none of these tests has been implemented routinely in clinical practice. A reliable readily available cost effective non-invasive test for endometriosis is yet to be established. The development of such a test remains one of the major priorities in endometriosis research.

This research aims to advance the understanding of miRNAs in the biological mechanisms underlying the long term systemic effects of endometriosis. Furthermore, given that miRNAs can be detected in blood according to the models of other pathologies, the study will investigate whether miRNA detection in blood can serve as a basis for establishment of a disease-specific non-invasive diagnostic test. Establishing such a test would have substantial benefit for women with the disease, their treating clinicians, and health service providers.

Better knowledge on menstrual cycle dependent alterations in circulating miRNAs in reproductive age women will improve understanding of the physiology of miRNA regulations. Additionally it will have utility for biomarker research, since there is a possible impact of the menstrual cycle stage on performance of any miRNA based test, with the possibility of preferable test timing in women across the menstrual cycle.

1.4 Hypotheses and objectives

Considering the material presented above, we hypothesized:

1. Endometriosis is associated with a unique plasma miRNA profile.
2. Plasma miRNAs are regulated in a menstrual cycle phase dependent manner.
3. Circulating miRNAs have a role in the development of endometriosis and may mediate systemic effects of the disease.
4. Endometriosis is associated with a unique miRNA profile in eutopic endometrium that is concordant with the endometriosis-specific signatures in plasma.

5. Circulating miRNAs can underpin a non-invasive diagnostic test.

In order to test these hypotheses, we formulated the following objectives:

1. To profile plasma miRNAs in a group of reproductive age women across the menstrual cycle to assess whether the levels of circulating miRNAs fluctuate with the cycle stage.
2. To profile plasma miRNAs in a group of women with and without endometriosis to determine whether the plasma miRNA expression pattern modulated by endometriosis.
3. To assess the possible biological functions and pathways linked with target genes regulated by the circulating miRNAs using computational approach.
4. To profile miRNAs in eutopic endometrium in women with and without endometriosis and correlate this profile with that observed in plasma.
5. To validate the endometriosis-specific miRNA profile in a larger-scale study to determine diagnostic accuracy and progress towards establishing a miRNA-based non-invasive diagnostic test for endometriosis.

Chapter 2
Experimental strategies for circulating miRNA research

2.1 Introduction

The utility of miRNA-based biomarkers in the clinical setting is strengthened by their remarkable stability and ability to be detected in a wide range of tissue sources, both in fresh and in archived material. On the other hand, an accurate quantification of miRNAs might be challenging due to their unique structure and biogenesis. This includes their short length, relatively low abundance (~ 0.01% of total RNA), high sequence similarity within miRNA families as well as the need to distinguish the premature precursors from the mature targets of interest (242-244).

Although most tissues are RNA abundant and yield high quality miRNA that is suitable for profiling studies, the minute amounts of RNA typically recovered from biological fluids (e.g. plasma and serum) hamper miRNA quantification. Circulating miRNAs are of considerable interest as biomarkers of disease because of the implicit non-invasiveness of obtaining clinical sample via venesection. Rapid advancements in molecular diagnostic technologies have resulted in introduction of a series of sensitive experimental and analytical tools to study the expression and biological function of miRNAs. However, a lack of consensus on the best performing experimental method and continuing inconsistency in the analytical approach is likely to account for the discrepant results that have been reported in the miRNA literature (242).

Bearing in mind that meticulous selection of the research methods improves the accuracy and reliability of the results, we chose to summarise the available knowledge on the advances and limitations of miRNA research, prior to presenting of our experimental data. This section will discuss each step in the pipeline leading to successful analysis of circulating miRNAs as well as the rationale behind the selection of the experimental approaches in the current project.

2.2 Quantification of circulating miRNAs

2.2.1 Pre-analytical variables and sample preparation

Circulating miRNAs present in serum and plasma are presumed to be remarkably stable and resistant to degradation (245-248). However, miRNAs can be degraded under experimental conditions during sample preparation. Moreover, the reliability of miRNA detection can be also compromised by degradation of total RNA that affects both the gene expression analyses (249-250) and miRNA profiling (251). Therefore, the sample collection procedure, RNA

stabilization, RNA isolation and further downstream processes, which contribute to RNA integrity, consequently influence the quantitative miRNA analysis (242-244, 249, 252-255).

2.2.1.1 Sample collection, processing and storage

Several variables associated with the blood collection may plausibly affect the amount and quality of miRNA recovered from a given plasma or serum sample (244, 253, 256-257). Blood cell lysis and contamination of the sample with the skin plug obtained at the skin puncture can contribute a significant amount of confounding miRNAs due to a cellular carryover, as cells have a much higher concentration of miRNA than plasma or serum. In our experiments, blood was collected by 21-gauge needles, which are usually employed to draw blood for testing purposes. All samples were processed by the same operator under the similar centrifugation conditions and special care was taken to avoid carryover from the cellular pellet. Haemolytic samples, detected by the colour of the supernatant, were discarded.

Pre-analytic variables affect miRNA content (252), although reports on the influence of most of these variables on miRNA quality are still lacking. Such factors include a diurnal variation in miRNA levels, fasting vs. non-fasting state at blood collection (e.g. fatty meals can cause lipaemia that could affect RNA extraction efficiency) and blood cell counts. Therefore, in the absence of such data, it is prudent to try to match as many variables as possible in the collection and/or selection of case and control samples for research studies. We did not adjust for these parameters in our study; however blood collection for the verification phase of this study (Chapter 7) was performed from fasted individuals.

It is also important to consider the type of the tubes for blood collection and transportation. For plasma samples, anticoagulants can affect the performance of the downstream profiling. Heparin cannot be removed during RNA extraction; it binds to calcium and magnesium in the polymerase chain reaction (PCR) Mastermix and potently inhibits subsequent PCR. Citrate can be used for circulating miRNA quantification; however, it causes dilution of plasma and may trigger haemolysis. EDTA is therefore the anticoagulant of choice for collection of plasma samples for miRNA experiments (244, 252). For the collection of blood for generating serum, at least two alternative types of tubes are in common use: a simple tube in which the clotted blood is simply centrifuged with recovery of supernatant (serum), alternatively a serum separator tube in which a gel is included that allows more discrete separation of the clot from serum during the centrifugation process. Although both are effective, it is important that the

type of collection tube used be standardized within a study to avoid confounding the results (252). For the purpose of our experiments, all samples were collected in EDTA collection tubes.

Additional consideration should include whether the duration of time taken between blood collection and processing of plasma or serum affects miRNA levels, e.g. via degrading RNases. Several research groups reported that miRNAs in plasma appear to be stable for extended periods at room temperature (at least up to 24 hours) (245-248), however this effect has not been universally established for all the investigated miRNAs. The majority of published studies included specimens that were processed to generate plasma or serum within 4 hours of collection. In the absence of solid data on this variable, it is prudent in designing studies to standardize conditions as much as possible with respect to the time elapsed between whole blood collection and processing for plasma or serum. All blood samples collected for the current study were processed within 8 hours of blood collection and were kept at +4^{o}C in the hospital or laboratory fridge; overall the samples were kept at room temperature for no more than 1 hour.

Storage of plasma or serum at -80^{o}C appears to be acceptable as freeze–thawing does not appear to influence the quantitative assessment of miRNA extracted, at least for the previously examined abundant miRNAs (247-248, 253, 258). A recent study demonstrated that storing of serum samples for up to 10 years at -80^{o}C did not impact on the integrity of miRNAs; moreover, miRNA levels were stable when plasma samples were stored at -20^{o}C for 2-4 years (259). Although the majority of the published studies include the analysis of specimens that are several years old, the effect of duration of storage on miRNA levels has not yet been carefully evaluated, and it seems best to match case and control specimens with respect to duration of storage as much as possible and unnecessary freeze-thawing should be avoided (244, 252). For optimal results from archived samples, care should be taken to select only samples that have been collected and processed according to the same sampling protocol. All plasma samples included in our study were frozen immediately after separation, stored at -80^{o}C, have not been exposed to repeat freeze-thawing and were processed within 2 years of storage.

2.2.1.2 Plasma vs. Serum

Plasma is the cell-free supernatant obtained after centrifuging of blood that has been collected in the presence of an anticoagulant. Serum is the cell-free supernatant obtained

after centrifuging blood that has been allowed to spontaneously clot, after being collected in the absence of anticoagulant. Both plasma and serum are acceptable types of specimen for circulating miRNA analysis. MiRNA measurements between plasma and serum were found to be highly correlated (245, 248, 260), whereas some observed that the quantification cycle (Cq) values from plasma tend to be slightly lower than for serum indicating a higher miRNA content (258, 261-262). In general, plasma is a better controlled and more time efficient method of preparation (immediate centrifugation vs. spontaneous coagulation), compared to serum. Also 10%-15% more plasma is obtained from the same amount of blood and there are minimal coagulation-induced changes, such as cell lysis and activation of the cellular components induced by fibrinogen. The main disadvantage of plasma over serum is the use of anticoagulants that may interfere with the downstream analytical methods. Given that a global comparison of miRNA expression between plasma and serum has not yet been performed, mixing specimen types within a study is not recommended. We used plasma samples in our experiments after recognising the advantages cited above.

2.2.2 MiRNA extraction

2.2.2.1 Challenges in extraction of circulating miRNAs

The classical mRNA expression studies demonstrated that different RNA isolation methods greatly influence the quality of the experiments, which also holds true for miRNA expression profiling (255, 263). Reproducible isolation of cell-free circulating miRNAs presents a challenge for a number of reasons. First, plasma and serum contain abundant proteins and lipids that can potentially interfere with sample preparation and the detection assay. Secondly, RNA purified from plasma can also contain chemical contaminants, i.e. reagents used for extraction, which interfere with the accuracy of the profiling platform, e.g. inhibit PCR efficiency. Further, the extracellular circulating miRNAs exist in 2 distinct physical states, within small membrane vesicles (215-216, 219-220) or in a free form, complexed with the proteins (218, 224-225). Even though both of these miRNA states exhibit remarkable stability and resistance to degradation from endogenous RNase activity (213, 264), different types of extraction can differently affect these compounds and result in different miRNA profiles. In addition, the yield of RNA from plasma or serum samples usually falls below the limit of accurate assessment by the routine methods, which calls for an alternative way to assess the efficiency of miRNA recovery.

2.2.2.2 Methods of miRNA extraction

The principles for isolating miRNA are similar to those used in isolation of total RNA except that are often modified to retain (and sometimes to enrich) the small RNA fraction (265). MiRNA isolation is carried out in three main steps: 1. lysis and denaturation, 2. organic solvent extraction, 3. RNA precipitation.

The first protocols were based on the use of Trisol reagent, a monophasic solution of phenol and guanidinium isothiocyanate, that simultaneously solubilises biological material, facilitates lysis of tissues, denatures proteins, removes most of the cellular deoxyribonucleic acid (DNA) and inhibits RNases. For serum and plasma samples, Trisol is substituted by a concentrated product, Trisol LS reagent, which is designed for the processing of liquid samples. After solubilisation, the addition of chloroform results in phase separation with the RNA remaining in the aqueous phase. RNA extraction is completed by isopropanol precipitation, followed by an ethanol wash and re-suspension of the RNA pellet.

Widely available commercial extraction kits typically combine a phenol/chloroform organic solvent step with a purification step that involves binding and eluting from a silica or glass-fibre column, e.g. MirVana PARIS (Ambion) or the miRNeasy kit (RNeasy Mini Kit; Qiagen, Inc.) reviewed in (242, 244, 255). Denaturing polyacrylamide gel electrophoresis (PAGE) for recovery and enrichment of small low molecular weight RNA involves substantial loss of RNA (266) and therefore is not routinely used for challenging samples, like plasma or serum. The traditional Trisol LS method without column purification has been reported to perform well with different profiling platforms, comparably to small RNA-enriched total RNA (253-254, 263, 267). The direct comparison between Trisol LS and column-based protocols in plasma demonstrated that although the Trisol protocol exhibited the highest extraction efficiency, the signal intensities differed between different methods depending on a type of detection platform (268). In our hands, the Trisol LS method with modifications to the manufacturers' protocol showed good performance. The detailed protocol as well as the steps of its optimisation and evaluation of efficiency will be presented in Chapter 3.

In order to improve miRNA recovery from clinical samples with very small starting amounts of RNA, a carrier RNA is added during the extraction procedure. The presence of a carrier, such as glycogen or bacteriophage MS2, enhances the efficiency of the RNA extraction (up to 2 Cq) and ensures a higher and more consistent RNA yield (248, 269). This was demonstrated in our experiments (Chapter 3) and our protocol included the addition of glycogen carrier.

The efficiency of the RNA extraction step can be assessed by addition of non-human synthetic miRNAs (e.g. from C. Elegans) that are not expressed in the tested biological samples (248, 252). The synthetic miRNAs are “spiked-in” in a known quantity in the initial phases of miRNA isolation after the denaturation of plasma or serum (to avoid degradation by RNases present in fresh samples). These synthetic templates then go through the entire RNA isolation process and are ultimately measured by quantitative reverse transcriptase PCR (qRT-PCR) in the final RNA elute, providing an internal reference for normalization to assess technical variations between the samples. This step is important to adjust for sample-to-sample variability in both protein and lipid content of plasma and serum, which could affect the efficiency of RNA extraction, and introduce potential PCR inhibitors. In our experience, each one of the synthetic templates performs similarly to the average of all 3 and can serve as independent normaliser (Chapter 3). We routinely use spiked-in synthetic miRNA cel-miR-54 to assess the efficiency of miRNA extraction.

Another important variable in RNA extraction is the expertise of the individuals who perform the experiment and how meticulous they are to prevent RNase contamination of the reagents and degradation of the RNA sample. Even slight deviations from protocol were reported to induce relatively large changes in experimental outcome (255). Our experimental results showed high concordance between the 2 operators with different levels of experience, when the protocol steps were carefully followed, indicating high reproducibility (Chapter 3).

Several new technologies have been recently introduced to the market. They avoid an organic separation step, hence minimising the sample contamination with phenol and exposure to hazardous chemicals. This includes spin column chromatography using proprietary resin or silica membrane as the separation matrix (e.g. Norgen’s Total RNA Purification Kit, Norgen Biotek Corporation, Ontario, Canada; NucleoSpin miRNA plasma, Macherey-Nagel GmbH & Co. KG, Düren, Germany) or magnetic bead-based technology (e.g. miRACLE isolation kit, Jinfiniti Biosciences). The efficiency and appropriateness of these methods still needs to be established.

2.2.3 RNA concentration and quality determination

2.2.3.1 Challenges in quantity and quality assessment of circulating miRNAs

Assessment of the quality and quantity of the extracted RNA is important for reproducibility and accuracy in miRNA-profiling studies, as miRNA quantification depends on high quality RNA (270). Classical methods for measuring the concentration of nucleic acids do not allow discriminating between different fractions of small RNAs. Fortunately, many profiling methods can be performed using total RNA and specific assessment of the miRNA population is not always necessary. However, this does not apply to plasma and serum samples, which usually have total RNA yields that are too low to be accurately quantified. The standard methods for measurement of the RNA yield and quality include measurement of optical density (OD) with spectrophotometry, use of automated capillary electrophoresis instruments, or use of denaturing gel electrophoresis (242, 244, 267).

2.2.3.2 Spectrophotometry

RNA purity and quantity can be measured photometrically by measuring the OD at different wave lengths: 230 nanometre (nm) (A230, absorption of contaminants & background absorption), 260 nm A260, (absorption maxima of nucleic acids), 280 nm (A280, absorption maxima of proteins), and 320 nm (A320, absorption of contaminants & background absorption). The spectrophotometric method to quantify RNA involves measuring the absorbance at A260 based on the RNA extinction coefficient (1 unit A260 = 40 ng/µl). The A260/A280 ratio provides an indication of RNA purity (1.8-2.0 suggests RNA of good quality), whereas A260/230 and A260/320 should be maximized as these represent the degree of background absorption and contaminants (265, 270).

The use of an RNA carrier in plasma and serum samples makes the measurements of RNA optical density non informative, since the signal from the carrier is too dominant relative to the other RNA species and shields the signal for the RNA of interest. Even if the carrier is not included during the isolation, the concentration of RNA recovered from plasma or serum is below the limit for reliable quantification with spectrophotometry, being represented by aberrant spectra (244, 252). Similarly, our experience showed that the spectrophotometric RNA quantification was not successful for plasma samples, demonstrating low A260/A280 ratios and unusual spectral patterns (Chapter 3).

2.2.3.3 Gel electrophoresis

A classical way to access RNA quantity and quality is to run a RNA sample on a denaturing acrylamide or 1-4% agarose gel to separate the different fractions of RNA subunits (5S, 18S, 28S). For RNA of good quality a 28S/18S ratio of 2.0 is assumed. The technique requires relatively high amount of input material, about 500 ng, and therefore is limited to the tissues with much higher concentration of RNA than that in plasma or serum. Moreover, the subjective interpretation of the gel images strongly depends on the experience and examination of the individual researcher and can hardly be compared between different users and laboratories (270).

2.2.3.4 Automated capillary electrophoresis

Today, lab-on-chip technology for automated capillary electrophoresis is state of the art and is recommended for standardized RNA integrity control. Different lab-on-chip instruments are commercially available like the 2100 Bioanalyzer (Agilent Technologies, Waldbronn, Germany) and the Experion (Bio-Rad Laboratories, Munich, Germany). Both devices are sensitive, highly reproducible and suitable for a reliable quality control of RNAs (270) . After the sample components are electrophoretically separated and dye is incorporated, their fluorescence, which depends on the size of each component, is determined against internal standards using the software tool. For visualization and better interpretation, an electropherogram and a virtual gel image are generated. The 28S/18S ratio is calculated by assessing the peaks recorded in the electropherogram and the bands occurring on the gel-like image. Additionally, to simplify the assessment of RNA integrity the instrument software calculates a numerical value: RNA integrity number (RIN) on the 2100 Bioanalyzer and RNA quality index (RQI) on the Experion. An RQI or RIN value of 1 represents almost fragmented and degraded RNA while an RQI or RIN of 10 represents intact non-fragmented RNA (271).

In miRNA-abundant tissues, the RIN value that is obtained for total RNA can be an indirect indicator of overall sample and, consequently, of miRNA quality (251, 270). However, this is not applicable for the challenging samples such as plasma and serum. A 2100 Bioanalyzer chip for small RNAs can analyse small RNA of less than 200 nucleotides and recognize fragments of 15–40 nucleotides as miRNAs. The concentration of miRNA is calculated as absolute amount in pg and as a percentage of a total small RNAs, however this does not estimate miRNA quality. The estimation of miRNA abundance by this method may only be accurate when overall total RNA integrity is very high. Small fragments of degraded RNA can reach the analytical range

analysed by the chip and therefore lead to an overestimation of the miRNA amount (270). For the same reason, spike-in synthetic templates to control for RNA extraction efficiency introduce significant bias to the Bioanalyzer readings. We used 2100 Agilent Bioanalyzer chips for small RNA on the non-spiked samples, as will be explained in more detail in Chapter 3.

2.2.4 Detection of miRNA expression

2.2.4.1 Challenges in profiling of circulating miRNAs

The short length of mature miRNAs and lack of a common sequence for universal primer-binding, such as a poly (A) tail, prevents sufficient annealing to traditional reverse transcription and PCR primers. Moreover, the short length and variance in GC content of miRNAs leads to a wide variance in melting temperatures for annealing reactions, creating miRNA-specific biases (272). Due to sequence homology between the mature and precursor miRNA forms and due to the high degree of similarity between the mature miRNAs within a family, miRNAs must be selectively detected in a background of other diverse RNA species to the accuracy of a single nucleotide. Finally, there can be sequence length variability in biological samples even for a single miRNA that results in co-variants (isomiRs) with different miRNA stability and mRNA targeting efficacy (242). In order to cope with the above limitations, a range of technical platforms was developed for miRNA research. Overall, due to the low yield of RNA from body fluids and difficulty to precisely measure miRNA content, a fixed volume of eluted RNA is routinely used for all downstream applications, rather than a fixed quantity of input RNA (248, 252, 269).

2.2.4.2 General types of miRNA profiling

Profiling studies generally include two major steps: a discovery phase and a validation step. The discovery phase typically includes large-scale miRNA profiling that aims to screen for differentially expressed miRNAs in normal and disease tissue with as many targets as possible. A following validation step is based on the less complex and more sensitive techniques for discriminative assessment of each of the selected targets.

2.2.4.3 Large-scale miRNA profiling

The majority of currently well-established high-throughput platforms for the large-scale miRNA profiling are based on three major approaches: 1. qRT-PCR, 2. hybridization-based methods and 3. next-generation sequencing (273-277). Each of these techniques is described below and summarised in Table 2-1.

Table 2-1 Large-scale miRNA profiling, comparison between the platforms. Adopted from Pritchard, 2012 (242)

Advantages	Disadvantages	Platform examples[1]	Sample required	Cost[2]
qRT-PCR based array				
Established method Sensitive and specific Can be used for absolute quantification	Cannot identify novel miRNAs Labour intense Medium-through put (less detectors per sample)	TaqMan TLDA microfluidic cards (Applied Biosystems) TaqMan open array (Applied Biosystems) Biomark HD system (Fluidigm) miScript miRNA PCR array (Qiagen) miRCURY LNA qPCR (Exiqon)	1 – 20 ng	$$
miRNA microarray				
Established method Low cost High-throughput	Lower specificity Cannot be used for absolute quantification Cannot identify novel miRNAs	GeneChip miRNA array (Affymetrix) GenoExplorer (Genosensor) MiRNA microarray (Agilent) Mercury LNA miRNA array (Exiqon) Sentrix array matrix and Bead Chips (Illumina)	30-100 ng	$
RNA sequencing: next-generation sequencing platforms				
Detects novel miRNAs High accuracy in distinguishing miRNAs with similar sequence, isomiRs High- throughput	Cannot be used for absolute quantification Substantial computational support needed	HiSeq 2000 (Illumina) SOLiD (ABI) GS FLX + (Roche) Ion Torrent (Invitrogen) MiSeq (Illumina)	100 ng	$$$

[1]This is not meant as a comprehensive list, but as a sample of commercially available platforms;
[2] The more '$' symbols there are, the more expensive the materials per sample are.

Even though each of these technologies is robust and reproducible, there is no absolute standard for miRNA expression profiling. Commercially available microarrays differ in the number of detectors, species coverage and sensitivity, therefore the results are not always concordant across the array platforms (242). Studies that compared different platforms found high intra-platform repeatability and comparability to individual qRT-PCR but lower inter-platform overlap between the lists of the differentially expressed miRNAs (275, 278-279).

2.2.4.3.1 qRT-PCR-based methods

This approach relies on reverse transcription of miRNA to complementary DNA (cDNA), followed by qPCR with real-time monitoring of reaction product accumulation. The reactions are carried out in a parallel high-throughput form that allows for hundreds of simultaneous qRT-PCR reactions to measure multiple miRNAs under the same reaction conditions. The common strategies used for priming the reverse transcription reaction to generate cDNA that have been adjusted for miRNA research (Figure 2.1) include enzymatic addition of a poly(A) tail (e.g. miScript miRNA PCR array, SABiosciences/Qiagen) and generation of a reverse transcription primer binding site using a stem–loop primer (TaqMan technology, Applied

Biosystems). A detailed description of these techniques, including pros and cons, is presented below in a section “Validation of miRNA profiling”. Commercially available customizable plates and microfluidic cards use pre-plated PCR primers that are typically distributed across multi-well dishes or alternatively across microfluidic cards containing nanolitre-scale wells. A PCR reaction is run on a thermocycler and the amplification values are calculated for the entire set of miRNAs, based on an intensity of fluorescence which is proportional to the amount of an amplified product. A complementary pre-amplification step can be used to enhance the sensitivity of the megaplex reactions and is considered a method of choice for PCR-based high-throughput miRNA profiling (280).

An additional strategy to improve the performance of such platforms is incorporation into primers of locked nucleic acids (LNAs), which are the synthetic RNA/DNA analogues characterized by increased thermo-stability, which help to standardize miRNA primer hybridization conditions (miRCURY LNA qPCR, Exiqon) (224). LNAs are RNA monomers with a modified sugar phosphate backbone that has a 2’-O-4’-C methylene bridge. The bridge increases the monomer’s thermal stability, reduces its flexibility, and increases the hybridization interactions of the base. When used with standard bases (A,C,G,T, or U), probes synthesized using LNA have greater thermal stability when compared to conventional DNA or RNA and form a stronger bond with the complementary sequence. The structures of LNA and RNA monomers are presented in Figure 2.2. LNA probes have a more rigid structure and hybridization strength that confers greater sensitivity and discrimination, which enables them to discriminate between highly homologous targets with a single base difference (281-282). LNA primer based qPCR profiling improves a sensitivity of the platform but is reported to have lower specificity compared to the stem-loop approach (276).

The main advantage of qRT-PCR based platforms is that it is a comprehensive, fast and affordable method that requires a small amount of input material. It is also easily incorporated into the workflow for laboratories that are familiar with qRT-PCR. This approach has been widely used for profiling circulating miRNAs (214, 225, 248, 283) and it seems to have higher sensitivity than microarray-based assays (274). In our experiments, we used commercially available microfluidic cards based on qRT-PCR TaqMan chemistry, considering the high specificity and need for low input amount of RNA of this technique.

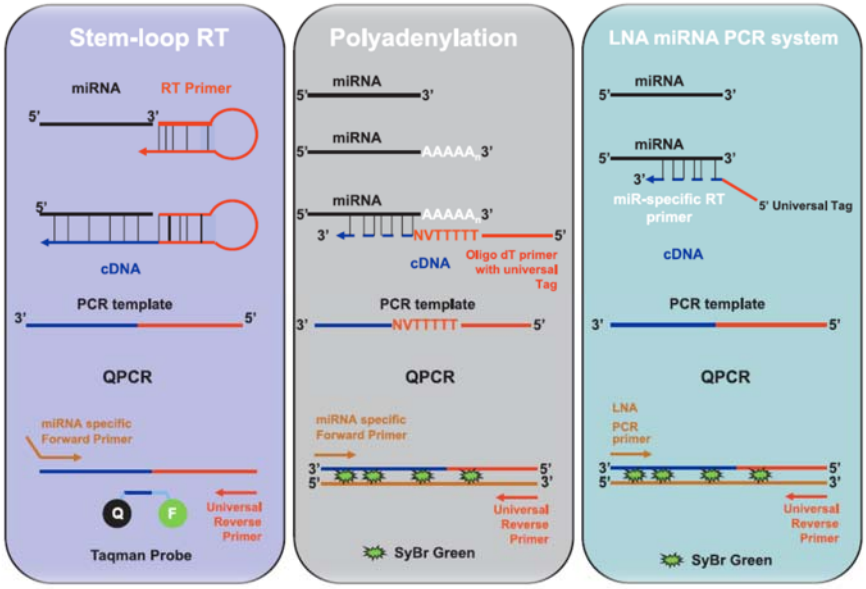


Figure 2.1 The three most commonly used methods for qRT-PCR quantification of miRNAs : a) Stem-loop primers, b) Poly(A) tailing of mature miRNAs, c) Locked nucleic acid –based primers. Adopted from Zampetaki A & Mayr M, 2012 (244).

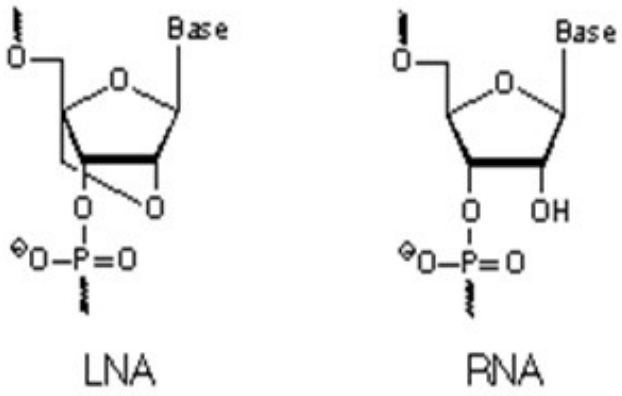


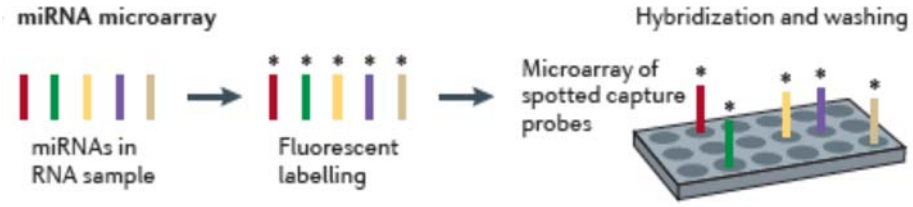
Figure 2.2 The structure of LNA monomer and RNA monomer. The key difference between RNA and LNA monomers is the Oxymethylene Bridge between the C20 and C40 atoms of the ribose ring. The resulting bicyclic sugar ring system imposes a locked RNA-like conformation.

2.2.4.3.2 Hybridization-based methods

Microarrays were among the first methods to be used for parallel analysis of large numbers of miRNAs. This method includes different techniques for fluorescent labelling of the miRNA in a biological sample (either by enzymatic or by chemical approach), followed by subsequent hybridization to DNA-based probes on the array or on beads. The expression of the targets is detected via scanning of the slides and quantification of the fluorescence, which is proportional to the target-probe hybridisation. Each of the labelling approaches has some limitations and benefits, but all may be associated with labelling of other RNAs in a sample,

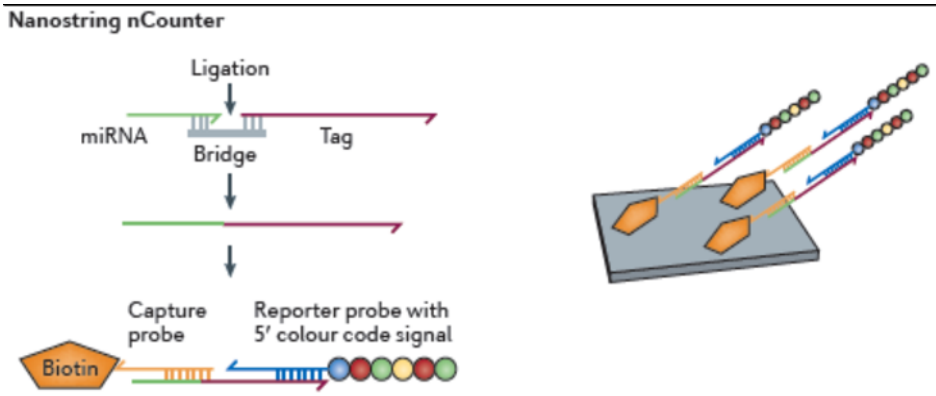
which can contribute to background signal as well as to cross-hybridization with specific miRNA probes. A recent innovation in miRNA profiling is the Nanostring nCounter system, in which a multiplexed probe library is created using two target-specific capture probes, a 3' capture probe containing biotin to allow absorbance to the solid phase via streptavidin and a second 5' reporter with an individual colour-coded sequence, that are tailored to each miRNA of interest. No amplification or labelling of miRNA is required with this method. The method provides high accuracy in discriminating between similar variants, but involves high costs and the distribution of the hard- and soft-ware supporting this technology is still limited. Microfluidic primer extension assays (MPEA) is another type of microarray in which DNA polymerase I is added directly into the channels for specific elongation and labelling of hybridised miRNAs, thus eliminating a labelling step before hybridisation (284). LNAs can be incorporated into capture probes on the arrays to improve the sensitivity and detection efficiency for miRNAs (267). Different methods of miRNA microarrays are illustrated in Figure 2.3.

Hybridisation-based miRNA microarrays have the advantage of being less expensive and yet they allow large numbers of parallel measurements. Their limitations include being more difficult to use for absolute quantification, having a lower specificity for closely related miRNAs, and requiring a larger minimum sample (~100 ng RNA) compared to qRT-PCR platforms. LNA and MPEA miRNA arrays require less RNA (30 ng and 20 ng, respectively), which makes these methods more feasible for the samples with minute amount of RNA (242, 244). Microarrays based on each of the abovementioned technologies have been successfully used for the profiling of plasma miRNAs and show good correlation with the qRT-PCR platform (261, 269, 285-287).



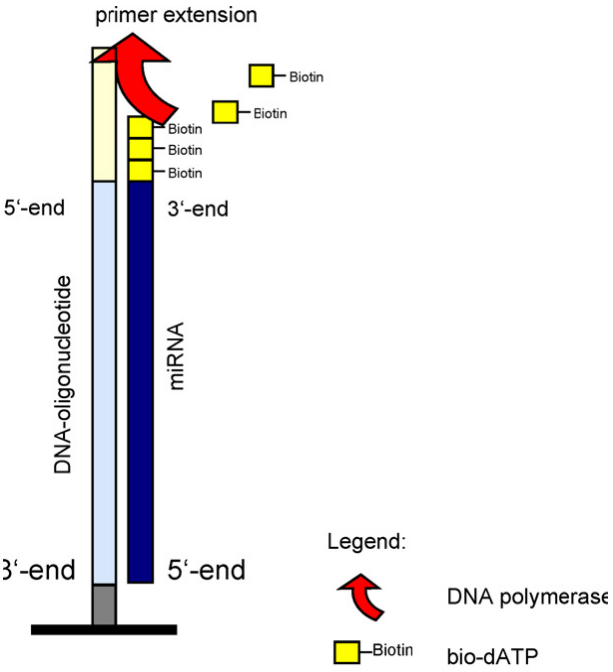
(A) miRNA microarray

DNA-based capture probes capture fluorescently tagged miRNAs, followed by scanning of slides and quantification of fluorescence



(B) Nanostring nCounter

A bridge oligonucleotide templates ligation of an miRNA to a specific tag. Capture and detection is done by two target-specific probes.



(C) MPEA

The hybridized sncRNA functions as a primer for an enzymatic elongation in which biotinylated nucleotides are incorporated.

Figure 2.3 Principles of miRNA microarrays: Panel A – hybridisation oligonucleotide microarray; Panel B - Nanostring nCounter; Panel C - Microfluidic primer extension assays (MPEA). Adopted from Pritchard CC et al., 2012 [Panels A, B] (242) and from Vorwerk S et al., 2008 [Panel C] (284).

2.2.4.3.3 Next-generation sequencing platforms, RNA-seq

This approach involves the reverse transcription of miRNA from the sample of interest to a cDNA library, which is then affixed to a solid phase (HiSeq 2000 or Genome Analyser IIX (Illumina)) or to beads for emulsion PCR (SOLiD (Applied Biosystems) and GS FLX+ (Roche)), as

illustrated in Figure 2.4. This is followed by the 'massively parallel' sequencing of millions of individual cDNA molecules from the library. Bioinformatics analysis of the sequence reads identifies both known and novel miRNAs in the data sets and provides relative quantification using a digital approach (a relative abundance is the number of sequence reads for a given miRNA relative to the total reads in the sample) (288). Currently established RNA-seq platforms begin with reverse transcription of miRNA to a cDNA library. Adaptor ligation then allows the library either to be affixed to a solid phase or to beads for emulsion PCR.

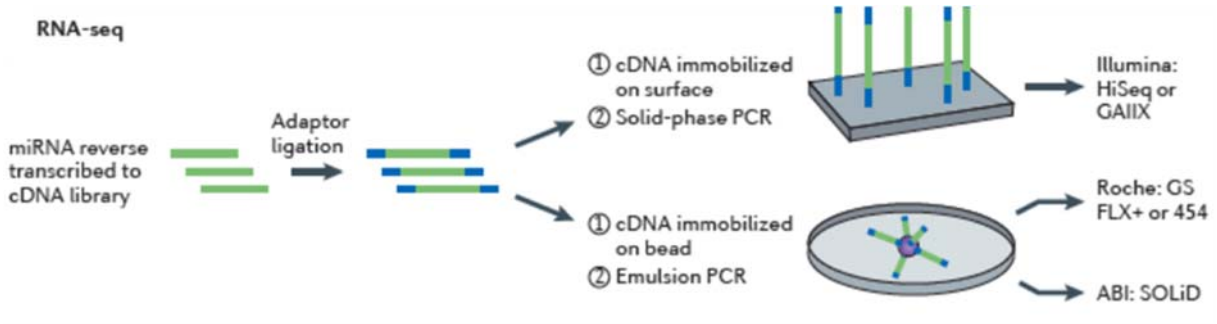


Figure 2.4 MiRNA profiling with next generation sequencing (RNA-seq). Adopted from Pritchard CC et al., 2012 (242).

The major advantages of next-generation sequencing for miRNA profiling is the detection of both novel and known miRNAs with precise identification of miRNA sequences (including sequences that differ by a single nucleotide and isomiRs of varying length), reviewedin (242). However, sequence-specific biases related to enzymatic steps in small RNA cDNA library preparation methods have been noted (289). Although next-generation sequencing technology has been projected to replace the use of the microarray in the near future (290), its current application is limited due to cost, labour, substantial computation and need for professional bioinformatics support (275, 279). Single-molecule real-time (SMRT) sequencing or single-molecule sequencing (SMS) methods promise faster and less biased output than methods that are currently in use, although they are not yet widely available and are associated with higher error rates and higher cost (242, 291). Next generation sequencing technology has been successfully applied to the measurement of circulating miRNAs (292-293).

2.2.4.4 Analysis and interpretation of miRNA microarray data

The processes that constitute basic miRNA-profiling data analysis can be summarized as 1. data processing and quality assessment, 2. data normalization, 3. calculation of differential expression and 4. tools for integration of mRNA and miRNA profiling datasets.

2.2.4.4.1 Data processing and quality assessment

The raw data is generated and exported by using the software incorporated into the instrument used for profiling (e.g. thermocycler). The data pre-processing includes platform and vendor specific steps, such as e.g., baseline adjustment and threshold setting for qRT-PCR analyses, background correction for microarray technology, or filtering for small RNA-sequence data. Investigation of whether any of the automated pre-processing steps can influence the analysis is important.

Quality assessment is essential for obtaining of valid and meaningful results and can be performed manually or automatically as an integrated algorithm in various data analysis packages. This step includes evaluation of the performance of internal controls, assessment of batch to batch variability and identification of outliers. Data quality control across samples can be assessed via diagnostic aids such as density distributions, box plots, scatter plots and histograms to provide a domain-relevant insight into the data. The plotting of $\log_2$ intensities or Cq values allows visualisation and identification of any substantial artefacts and systematic patterns (294).

Negative controls, including no template controls (NTC) should exhibit weak or no amplification, to demonstrate the absence of contamination by DNA. The performance of an internal reference (standard sample or template that is run across different batches) can help to estimate the overall performance of each array as well as the batch effects related to run-to-run variation. The batch effects can be minimized by making sure that each batch of samples includes a mix of samples from the different comparison groups (295).

The signal-to-noise ratio (SNR) is a statistical tool that measures the quality of the signals that are obtained from hybridisation arrays. When SNR is low, the background noise could dominate the measured expression signal and thus increase the uncertainty in evaluating gene expression levels. The SNR is not computed for qRT-PCR based arrays due to lack of estimates for the background noise on these platforms (279).

The analysis of technical replicates (i.e. repeated measurements of the same sample in the same run) minimises variation bias and permits removal of outliers prior to aggregation of replicates into a single measurement value. The applicable replicate variability threshold is 0.3-0.5 Cq in qRT-PCR based methods. The biological variability is often much larger than the technical variability; however it is not advisable to remove any of the biological replicates unless the sample size is sufficiently large (295-297). A principal components analysis (PCA)

and an array-array heatmap based on the Spearman distance that is produced as part of the results, allow post-facto review of quality of the experiment. Finally, it is important to ensure that a data-handling error has not occurred during the uploading in the analysis software or spread sheet, particularly after merging of multiple data files (242). Quality assessment algorithms that control for the above parameters are incorporated in commercially available software (e.g. REST, qBASE, StatMiner) and in the Bioconductor packages designed for gene expression and miRNA analysis.

2.2.4.4.2 Normalisation methods

Data normalization is crucially important for obtaining accurate results when comparing different samples and conditions and is known to have a profound impact on experimental accuracy (242, 295, 298-300). The goal of normalization is to adjust the data to minimize the effects of systematic experimental bias and technical variations, thereby making it easier to identify the relevant biological differences. Various approaches have been used in the literature, and some of the discrepancies between miRNA-profiling studies may be explained by the application of different normalization approaches.

The first microarray experiments employed no normalisation, using background subtraction followed by normalizing to a median or scaling based on total array intensities (266, 299). More recent approaches range from the relatively simple methods to more complex algorithms and most of them are suitable for several experimental platforms.

Perhaps the most common approach for normalization of qRT-PCR data is the use of one or a few invariant endogenous controls or “housekeeping” reference miRNAs that are found to be largely stable across the samples (252, 300). This approach adjusts the data for differences in input RNA as well as differences in sample quality that may influence the efficiency of qRT-PCR. The danger of using a priori selected “predefined” endogenous controls lies in the assumption that a specific miRNA exhibits exactly the same pattern of expression in all sample types independently of the experimental condition. At present, there is no consensus regarding universal specific miRNAs or a set of miRNAs that can be used as reliable endogenous controls (298, 300). The factors that lead to biological variation are not known, and their effects on specific miRNAs in different sample sets have not been characterized. Gene expression analyses have shown that many genes assumed to be constant in their expression can vary between conditions (301-302). Thus, selection of normalisers that are specific to the experimental sample set outperforms the normalization strategy that uses

predefined universal endogenous controls, such as the small RNAs proposed by the manufacturers (e.g. Mammalian U6) (298, 303-304).

Whereas a single miRNA normaliser may be sufficient in some experimental situations, a geometric averaging of more than one may be required to produce accurate data. The strength of using geometric averaging is in smoothing the individual variation of the expression value of a single reference gene, which reduces the errors in normalized data (305). Selection of invariant miRNAs from the experimental dataset can be identified by algorithms specifically developed for reference gene or miRNA evaluation and selection. Examples of algorithm to identify normalisers include: 1. GeNorm based on the ranking of the putative controls and stepwise elimination of the least stable ones (305), 2. NormFinder based on statistical linear mixed-effects modelling (306) and 3. BestKeeper, based on repeated pairwise correlation and regression analysis (307). The optimal number of stable controls is recommended to be determined by a pairwise variation analysis between subsequent normalization factors using a cut-off value of 0.15 (305). Ideally these candidate references should belong to different functional classes, significantly reducing the possibility of confounding co-regulation (298). Only the miRNAs that are expressed across all the samples are commonly used for a screening search for stable references.

An alternative approach that has been used for large-scale miRNA-profiling datasets is to use a global measure of the miRNA expression data as the normaliser. This strategy is based on the assumption that although specific miRNAs may vary across biological samples, the overall pattern of expression of the majority of miRNAs is expected to be invariable and therefore a global measure of expression can be used to correct for technical and/or sample quality differences. Several authors have discussed approaches along this line (298-299, 303, 308-309). The most commonly investigated approaches include the normalisation to Global Mean expression, Locally Weighted Regression and Smooting Scatterplots (LOWESS) normalisation, variance stabilizing normalization (VSN) and Quantile normalisation.

The Global Mean expression applies a normalisation to the mean expression of all miRNAs that were found to be expressed in a given sample (303). The approach has been found superior to normalisation to a range of endogenous controls in qRT-PCR based arrays and was validated using multiple data sets, including those from hybridisation microarrays (309).

The VSN is based on a parameterized arsinh transformation (instead of a logarithmic transformation) that calibrates sample-to-sample variations and renders variance

independent of the mean intensity. However, VSN strongly affects the distribution of the large fraction of miRNAs whose expression is near or at background, resulting in the large increase of variability for those miRNAs (295, 299).

LOWESS normalizations and its variants are widely preferred transformation based methods, which use local regression via locally weighted scatter plot smooth (295, 308). This method corrects for intensity-specific artefacts of the spots/channels on each array that can be visualised via plot of the spot intensities vs. an average brightness of the chip. The LOWESS detects the systematic deviations on the plot and corrects them by carrying out a local weighted linear regression as a function of the log10 (intensity) and by subtracting the calculated best-fit average $\log_2$ (ratio) from the experimentally observed ratio for each data point.

Quantile normalization assumes that on average, the distribution of the transcript levels within the cell remains nearly constant across samples, so that if the expression of one transcript increases, that of another decreases. A quantile is a measure that allows assessing the degree of spread in a data set, which is divided into n-regular intervals, which are equivalent to the number of data points. The algorithm equalizes the distributions of expression intensities across arrays by fitting the intensities of all probes on each array into one standard distribution shape and then maps every value on any one array to the corresponding quantile of the standard distribution by assigning rank order for each value (310). Originally proposed for oligonucleotide arrays by Bolstad et al., 2003, it is now widely used for different platforms and was confirmed as one of the most robust methods (298-299, 301, 311-312). This method works as well as most of the more complex procedures, and is available in affy, qPCRNorm and other packages distributed through the Bioconductor project (www.bioconductor.org), which has become the most widely used suite of freeware tools for microarrays (294, 301).

LOWESS and Quantile algorithms assume that most targets are not differentially expressed and differentially expressed spots are homogeneously distributed with respect to both, over- and under-expressed miRNAs. These assumptions may not hold true in case large numbers of miRNAs are differentially expressed or expressed in only one direction, in which case algorithms that make only the minimal (e.g. stable control-based) or no assumptions (e.g. VSN) might be more appropriate for the respective datasets (298-299).

A third approach is the use of spiked-in synthetic control miRNAs that are introduced into the RNA sample at a range of known input amounts and that are not normally present in the sample (252, 295). Mitchell et al. (2008), introduced the concept of spiked-in synthetic non-human miRNAs, which are added to a plasma sample prior to extraction (248) to adjust for differences in efficiency of RNA recovery between samples and as internal normalisers for downstream PCR quantification. The lack of the sequence homology of such miRNAs to human miRNAs and absence of empiric hybridization to human miRNA probes on miRNA microarrays made this approach particularly attractive. This method has the advantages of providing rigorous quality control, correcting for many aspects of technical variation (for example, sample-to-sample differences in miRNA-labelling efficiency for microarrays or reverse transcription for qRT-PCR-based profiling). It requires that assays for the spiked-in controls should be available on the platform that is being used, however their use does not control for technical variations other than process-related losses of miRNA abundance (266).

For miRNA profiling by next-generation sequencing, normalization methods are much less well-developed (298). Currently, a common approach is to represent the abundance of a given miRNA in a sample as the percentage of total reads. This approach, however, assumes similar distributions of overall miRNA reads between the samples, which might not be true. In the future, the development of a statistical framework for adjusting for differences in miRNA frequency distribution, and the use of synthetic miRNA spike-in approaches may provide improved data normalization between samples and improved quantification ability in RNA-seq experiments.

It remains unclear, which method uniformly outperforms the others under different biological context and if there is any single approach for different experimental set ups. Evaluation of several normalization strategies to find the optimal one for the dataset in consideration or even a combination of two normalization methods have been suggested as more robust approach (277, 299, 308, 311). We applied several normalisation approaches to data obtained from the array experiment. Quantile method appeared to outperform other techniques by more significantly reducing the variability measures within the data, as demonstrated in Chapter 4.

2.2.4.4.3 Calculating differential expression and determination of statistical significance

Depending on whether or not annotation to identify the experimental groups is used, the analyses can be classified into supervised or unsupervised learning. In unsupervised learning, data is classified into groups with similar patterns, i.e. similar expression profiles, without giving a priori annotation to the groups. Clustering methods is one of the widely used unsupervised approaches and can be hierarchical (grouping objects into clusters by specifying relationships among objects resembling a phylogenetic tree) or non-hierarchical (grouping into clusters without specifying relationships between objects in a cluster) (313).

Supervised learning in miRNA-profiling experiments involves making comparisons between the predefined groups, i.e. disease and normal state, typically by the calculation of differential miRNA expression. The data from the profiling experiments is usually presented as a relative measurement and the absolute units are not necessary to assess up- or down-regulation across two different conditions (313). In addition to the relative expression values, the data can be presented by using the fold-changes (FC) between the conditions. In such cases, a ratio of 4000/100 will lead to the same result as 40/10, which does not provide any information on absolute measurement, but represents a meaningful comparison across different conditions. The degree of fold-difference that constitutes meaningful differential expression will depend on the experimental context. An acceptable recommendation FC cut-off is 1.5, but can be raised up to 5 if more than 5% of the targets are selected, in order to direct further investigation on the limited number of differentially expressed targets (314). The FC can be represented as $\log_2$ values, which allows mapping of up- and down-regulation and down-regulation in a symmetric manner, e.g. no difference is presented as 0 (log2 1 = 0), up-regulation as a positive value ($\log_2 40/10 = 2$) and down-regulation as a negative value ($\log_2 10/40 = -2$).

Among multiple statistical methods, LIMMA (linear models for microarray analysis) is considered as highly robust for differential expression analysis under varying experimental designs. LIMMA uses a moderated t-statistic that allows “borrowing information from the ensemble of genes which can assist in inference about each gene individually” (315). LIMMA has improved power over other approaches for low sample sizes, is tolerant of data having both normal and some non-normal distributions and supports both blocked and non-blocked designs and is useful for ranking genes by likelihood of differential expression (315-316).

Several studies that assessed a large number of statistical methods, found that the method implemented by LIMMA performs well for both large and small numbers of samples and has been proved to be much more reliable than other examined methods, including Significance Analysis for Microarrays (SAM), Analysis of Variance (ANOVA), the T-test, VarMixt, RankProd, and others (317-320).

Every statistical test is related to a single hypothesis per detector and per experimental condition comparison, thus the number of tests is equal to the number of miRNAs in the project. Increasing the number of tests increases the probability of finding a significant p-value by chance. Generally, a correction for multiple comparisons requires raising the bar for significance from what would be called significant for a single test (p value = 0.05) to a level dependent on the number of tests being run in parallel. This minimises the effect of the False Discovery Rate (FDR), which is defined as the expected proportion of false positives among the declared significant results. There are several methodologies to implement the correction for multiple testing which negatively adjust the p-values according to the number of total tests. The most popular is the Benjamini-Hochberg (B-H) method that assumes that the multi-tests are dependent and that the expression of the detectors has some level of correlation (321). Other methods to adjust for multiple testing include Bonferroni, Hochberg or Holm's methods. Therefore, in high-throughput analysis targets are usually filtered for statistical significance using FDR testing, with an industry-standard FDR-adjusted p-value cut-off of 0.05. However, if less than 0.1% of the expressed targets are found as differentially expressed, the raw p-values without correction for multiple testing can be used to rank genes by likelihood of differential expression (with a cut-off of 0.05). Some experts believe that correction for multiple testing can be omitted in a discovery type of experiments (322). In such experiments, the main objective is to generate the hypothesis that needs to be followed up further. For this reason, producing too many type 2 errors by applying a multiple test correction can deter potentially exciting observations, some of which may later prove to be false. In this scenario, the authors have to be clear in setting objectives and cautious in interpreting the results. We used LIMMA for statistical analyses of micro array experiment and, in accordance to the presented above, did not apply a correction for multiple testing as a mandatory criterion for the significance of differential expression in this discovery stage.

The major analytical challenge associated with analysis of the circulating miRNAs and their association with disease is that the circulating miRNAs are highly correlated, which affects the

precision of conventional statistics. This might lead to over fitting and potentially biased effect estimates. Application of the computational networks to miRNA data that account for the relationships between miRNAs as well as between miRNA clusters and pathologic conditions, have been proposed. The utility and methodological approach of such algorithms is currently under investigation (323).

2.2.4.4.4 Integrative analyses of miRNAs and mRNA profiles in gene regulatory networks

A major area of interest in miRNA-profiling analysis is integration of miRNA profiles with mRNA profiles and other large-scale genomic data sets (324). This has the potential to yield a better understanding of gene regulation and to improve systems-level modelling. *In silico* tools are increasingly becoming available for the functional analysis of altered miRNA subsets and for interrogation of miRNA regulatory networks (325-329). The overview of current bioinformatics approaches towards identification of the biologically important miRNA targets, including assessment of their limitations, is summarised in several recent excellent reviews (330-331).

2.2.4.5 Validation of miRNA profiling

miRNA microarrays are best used as discovery tools rather than as quantitative assay platforms, thus independent confirmation of microarray results is generally advisable for the key findings in every experiment (332). The validation can be performed using additional microarrays, qRT-PCR, northern blot, or other techniques. QRT-PCR remains the gold standard for quantification of gene and miRNA expression. It has been widely employed as a validation method for microarray studies, due to its wide dynamic range, high accuracy and ability to provide absolute miRNA quantification (278, 296, 304). The efficient cDNA synthesis methods, integration of amplification efficiency, inclusion of suitable internal controls, rational outlier exclusions and sufficient replication are the bases for the accuracy and reproducibility of results.

2.2.4.5.1 Synthesis of cDNA

There are several different methods to reverse transcribe miRNAs (304). MiRNAs can be reverse transcribed individually by using miRNAs-specific reverse transcription primers (MSPs). Alternatively, miRNAs are first tailed with a common sequence and then reverse transcribed by using a universal primer. As a general rule, the use of MSPs decreases

background, whereas universal reverse transcription is useful if several different amplicons need to be analysed from a small amount of starting material. Another alternative can be to multiplex miRNAs reverse transcription by pooling MSPs. The resulting cDNA is then used as a template for qPCR with one MSP and a second universal primer. While the 3'-end of the MSP has to be complementary to the miRNA, there are two different approaches to design the 5'-end of a MSP: with either a stem-loop or a linear structure. Stem-loop primers (used in TaqMan technology) are designed to have a short single-stranded part that is complementary to the 3'end of miRNA, a double-stranded part (the stem) and the loop that contains the universal primer-binding sequence. Stem-loop primers are more difficult to design but their structure increases specificity of the assay by reducing annealing of the primer to pre- and pri-miRNAs. The 3'-end of linear primers is designed to complement the target miRNA, to enable reverse transcription, while the 5'-end of the primer encodes a universal sequence that is used to achieve qPCR amplification. A main disadvantage of linear primers is that they may not discriminate between mature miRNA and their precursors. Incorporation of LNAs into a primer increases sensitivity of the assay by increasing melting temperatures, but contributes to a higher cost.

Out of a number of fluorescent technologies available for qPCR, two are used for miRNA detection; SYBR Green and TaqMan probes. The SYBR Green chemistry is based on the increase of the fluorescent signal proportionally to the accumulation of the amplification products, but cannot discriminate between different PCR products and binds to all double stranded DNAs, including non-specific products such as primer-dimers. Therefore, methodologies that assess the specificity of the amplification products, such as a melting point analysis, are often required to monitor the homogeneity of the qPCR products. TaqMan primers incorporate the dual-labelled hydrolysis probes that contain a fluorescent reporter and quencher upon adjacent nucleotides. The close proximity of the fluorescent reporter to its quencher molecule prevents the emission of fluorescence. During the amplification process, the primer loop unfolds upon denaturation and Taq polymerase hydrolyses the probe. This results in increased distance from the quenching group and in increase of fluorescence, proportional to the amount of the amplicon. Given that a non-specific amplification will not generate any fluorescent signal, this method allows more accurate quantification of the amplified target.

2.2.4.5.2 Normalisation of RT-PCR data

As with high-throughput analysis, the reliability of the validation miRNA experiments requires appropriate normalisation. For cases in which a small number of miRNAs are being profiled, the approaches based on global measures (e.g. quantile or a global means of expressed miRNAs) are not a suitable strategy. In such cases, an appropriate method is normalisation to the specific endogenous controls, which are identified in a pilot large-scale profiling study on a subset of samples using one of the abovementioned methods (297, 301, 303). The use of microarrays to identify candidate internal references for qRT-PCR normalization has been successful, but this extrapolation requires some caution due to differences between the microarray and validation experiments. The main source of discrepancy is likely to be due to differences in the types of the samples, in the platform design or in data transformation procedures during normalization. As even small variations of an internal control could lead to non-reliable expression data, it is critical to validate that the expression of reference miRNAs is stable prior to their use for normalization in qRT-PCR analysis (302). However, testing of the expression stability of the putative normalisers for the circulating miRNAs in qRT-PCR experiments can be challenging. This is because this type of experiments is based on the input volume and not the exact amount of miRNA, unlike gene- or miRNA-abundant tissue studies. Normalisation by using synthetic miRNAs spiked-in as controls has also been described (248, 252), however this approach is not as accurate as appropriately selected internal references.

2.2.4.5.3 Real time quantification

2.2.4.5.3.1 ΔΔCq vs. efficiency-calibrated method

The principle of qRT-PCR is based on the detection, in real-time, of a fluorescent reporter molecule whose signal intensity correlates with amount of DNA present in each cycle of amplification. qRT-PCR exploits the fact that the quantity of PCR reagents is not limited in the exponential phase, therefore PCR product increases exponentially in proportion to the quantity of initial template. Ideally, PCR product will double during each cycle if efficiency is perfect, i.e. 100%. The cycle number at the threshold level of log-based fluorescence is defined as Cycles to Threshold (Ct), more recently termed as the Cq number. This is the observed value in most qRT-PCR experiments, and therefore the primary statistical metric of interest.

Real-time PCR data are quantified absolutely and relatively. Absolute quantification is important if the exact transcript copy number needs to be determined, however, relative

quantification is sufficient for most physiological and pathological studies (333). Two mathematical models for calculation of the relative expression are widely applied: the efficiency calibrated model (334) and the ΔΔCq model (335). Both methods are based on similar experimental models and rely on the comparison between expression of a target miRNA versus a reference miRNA. The ΔΔCq method involves two subtractions: the first between the endogenous control expression value from the target of interest measured in the control sample and the second, the endogenous control in the experimental sample from the target of interest measured in the experimental sample (335). Comparison between the two conditions is possible by comparing normalized expression (ΔCq) of the two conditions or by calculating the fold change of the expression, i.e. the expression ratio, (ΔΔCq). The ΔΔCq method assumes the percentile amplification efficiency (PAE) is 100% and calculates the ratio based on $2^{-\Delta\Delta Cq}$, where 2 corresponds to a doubling of PCR products per cycle. However, this approach does not take into consideration possible variations of amplification efficiencies from one sequence or sample to the other and thus may not accurately estimate relative DNA amounts in different conditions.

The efficiency-calibrated model assumes that different targets are not amplified with the same efficiency, thus the comparison of their expression levels requires some adjustment. PCR amplification efficiency can be either defined as percentage (PAE) (from 0 to 100%) or as time of PCR product increase per cycle, i.e. amplification efficiency (AE) (from 1 to 2). The AE can be estimated by the analysis of the standard (dilution) curves, by individual graph analysis based on the rate of fluorescence accumulation within the exponential region or by mathematical model fitting (336). The standard curve method calculates the AE based on Cq numbers from a serial (usually 5-log) dilution of templates, via plotting of the Cq values versus the log of the dilution and applying a linear regression (337). For each dilution, a standard qRT-PCR protocol should be performed in triplicate for all the primer pairs to be used in the experiment. The equation of the linear regression line, along with Pearson's correlation coefficient (r) or the coefficient of determination (R^2), can then be used to evaluate whether the qPCR assay is optimized. $AE = 10^{-1/slope}$ and $AE = 2^{PAE}$, thus $PAE = \log_2 AE$ (336). Standard deviation (SD) on the efficiencies calculated with the standard curve are evaluated from the SD of the slope of the regression using a Taylor expansion to the first order for error propagation: $|\Delta AE| = \sqrt{(AE \times \ln 10 \times \Delta m)^2}$, where ΔE is a SD of the efficiency, and Δm is the SD of the slope of the regression (337).

The original formula implemented in the efficiency-calibrated model is: a relative fold change = $AE^{-\Delta Cq\ target\ (sample - control)}$ / $AE^{-\Delta Cq\ reference\ (sample - control)}$ (334). A more flexible approach introduces adjusted Cq values, in which the raw Cq values are adjusted for amplification efficiency to the level they would be if they had been amplified at 100% efficiency (336-337) as following: adjusted Cq = Cq x $\log_2(AE)$. Then standard ΔΔCt method formulas can be applied by using the standard formulas: relative expression = $2^{-Cq\ (target\ -\ reference)}$, fold change = $2^{-\Delta Cq\ sample\ (target\ -\ reference)}$ / $2^{-\Delta Cq\ control\ (target\ -\ reference)}$. Even though proper efficiency adjustments can often improve the data quality, the standard ΔΔCt method may be a method of choice for the discovery stage of the experiment, allowing for quick assessment of large amount of targets.

2.2.4.6 Statistical analysis of RT-PCR data

Intra-assay variation can be calculated for every single sample of every reaction if the real-time PCR experiments are performed in triplicate, with a pooled variance for all sets of PCR triplicates representing statistical power. Inter-assay variation should be quantified in cases where comparisons are made of results from separate assay runs and can be performed using data from either a internal control or standard sample that are included on all plates (338). KOD and Grubbs' test have been proposed as outlier exclusion methods and can be integrated for data analysis (336). Several methods to overcome problems with missing values can be applied: 1. discard unexpressed targets to avoid false results, 2. set undetermined values to a maximum Cq (e.g. 40), 3. perform an imputation if the Cq value is totally absent (an imputation is performed by using the values of the other biological replicates via selection of the median as aggregation method between samples with the same experimental condition).

Not many publications deal with the statistical analysis of expression data generated by qRT-PCR. Common statistical methods include parametric tests, such as the Student's t-test or ANOVA or non-parametric tests, such as the Mann-Whitney U-test or the Kruskal-Wallis test (339-341). The assumptions of normality of PCR data can be violated since the relative expression values are derived from the ratios and not absolute values, or because the experimental design of most PCR experiments is confined to a limited sample size. When normality is not proven, using a non-parametric test (not assuming normality) reduces the risk of misinterpretation of the results. However, a non-parametric test is less powerful than parametric test when the data are normally distributed. Logarithmic transformation is recommended for expression analyses, mainly because an accurate estimation of the distribution of the Cq values might be challenging and this transformation gives the data a

"normal" distribution (339, 342). An alternative suggestion is to assess qRT-PCR data in a linear scale first, i.e. to convert Cq values to the relative expression values. This is because raw Cq values are logarithmic in nature and therefore grossly underestimate the true degree of variation that would be evident if the Cq values were converted into linear values (343). High Cq values tend to exhibit higher variance (344), whereas the same change in Cq with increase in Cq values means a different raw change in the number of initial copies represented and therefore a different associated variance/error. Moreover, the error is not equal above and below each bar on log- transformed qPCR data plotted on bar graphs due to this.

2.3 Guidelines for publication of the expression profiling data

Given the highly dynamic nature of miRNA transcription and the potential variables introduced in sample handling and in the downstream processing steps, a standardized approach to each step of the analysis workflow is critical for reliable and reproducible results. This is assured via development of the universal set of guidelines to establish a solid experimental approach.

The Microarray Quality Control (MAQC) consortium began in February 2005 as a Food and Drug Administration (FDA) initiative with the goal of addressing various microarray reliability concerns raised in publications. The first phase of this project (MAQC-I) extensively focused on the technical aspects of the profiling experiments with the microarray platforms (345), while the second phase of the project (MAQC-II) has focused on the multivariate gene expression–based prediction models (297). MAQC does not include straightforward FDA recommendation for regulatory submissions, but rather provides useful general guidelines for planning, conducting and evaluation of high-throughput experiments.

The Minimum Information for Publication of Quantitative Real-Time PCR Experiments (MIQE) provides a standardized approach to each step of the qRT-PCR workflow towards generation of the reliable and reproducible results, providing guidance for assessment and presentation of PCR data (346-347).

Finally, all miRNA-profiling data has to be accessible to other researchers, particularly for metaanalyses, in which combining of several experiments helps to increase power without further costly experimental work. The data needs to be available to the reader in both raw and normalized fashions; multiple experiments and multiple readings for the same probe must

be made available without averaging. This can be done by addition to public databases such as Gene Expression Omnibus (GEO) and the use of supplemental data but is often done best via peer-to-peer Internet sites (312).

2.4 Summary points

- Several technical aspects and pre-analytical differences in sample collection and preparation may influence the outcome of miRNA profiling studies.
- It is not clear which of the existent RNA purification techniques is technically superior; miRNA extraction appears to be detection-platform dependent.
- High-throughput assays are an initial discovery step in miRNA profiling. Several platforms are available and there is no absolute consensus on the preferred method.
- Simultaneous profiling of hundreds of targets introduces potential biases due to the complex data manipulations and thus the data require validation.
- qRT-PCR is the method of choice to validate the results of high-throughput analyses.
- Application of an optimal normalization method has a significant impact on the results of the profiling experiments. It remains unclear whether specific methods uniformly outperform the others; selection of the optimal normalization method should depend on the characteristics of the dataset. Normalisation based on global measures of expression is more robust for high-throughput datasets, whereas endogenous controls are a method of choice for the low-scale validation experiments.
- The optimal statistical treatment for qRT-PCR includes precise data modelling, integration of amplification efficiency and rational outlier exclusions.

Chapter 3
Materials and Methods

1.1 Establishment of tissue bank (blood and endometrial tissue samples)

3.1.1 Recruitment of participants

First, we established a system to recruit patients and to collect blood and tissue samples in order obtain material for the planned experiments. Overall, the experiments were of 2 designs: case–control and prospective cohort.

An initial case-control study included healthy women and women with endometriosis who had responded to an advertising poster. Women with endometriosis were identified from the list of patients awaiting surgery for diagnosis and treatment of pelvic pain. For those who agreed to participate in the study and satisfied the inclusion criteria, 3 appointments for blood collection were planned based on the day of the last menstrual period. Of note, blood from women with endometriosis was collected prior to surgery and samples were excluded if endometriosis was not surgically confirmed.

The cohort study included all women scheduled for surgery for suspected endometriosis based on clinical symptoms (pain, infertility or both). This was planned as a large-scale study, thus to optimise the process of recruitment we established a collaborative network with the nursing and medical staff in 2 clinical settings: the Women's and Children's Hospital, Adelaide SA, a tertiary university public hospital and the Burnside War Memorial Hospital, a private practice of gynaecologic laparoscopist and endometriosis specialist Dr. Susan Evans. We organised to meet with the staff in each of the above institutions to explain the project and to decide on the most efficient method of patients' recruitment that would not disturb the clinical work flow. As a result of the collaborative agreement, we developed a system to approach and to recruit the patients which was specific to each of the clinical settings.

An information flyer with a general outline of the project was designed for the patients attending private practice. This flyer did not substitute the original information sheet and consent form approved by the Human Research Ethics Committee.

Overall, we managed to create a tissue bank of blood and eutopic endometrial samples from more than 100 participants. This comprised blood at 3 phases of the menstrual cycle from 12 controls and 15 women with suspected endometriosis, an endometrial biopsy from 11 controls and 17 participants with suspected endometriosis and paired plasma-endometrial biopsy from 68 participants with suspected endometriosis. Both serum and plasma were

separated from each blood sample; serum was used for hormonal measurements to assess menstrual cycle stage, plasma was used for miRNA isolation. Each case of suspected endometriosis was further verified by surgery and subsets were classified as endometriosis or symptomatic controls. Staging of endometriosis was performed according to the rASRM classification system (47, 348). All samples were well characterised; obtained data included demographic parameters and general medical, gynaecological and family history.

Written informed consent was obtained for all participants. The study was approved by the HREC of the Women's and Children's hospital and the Burnside War Memorial Hospital (REC 2121/11/11).

3.1.2 Sample collection and processing

Each sample in our tissue bank was processed and stored under similar conditions in accordance with the appropriate well established protocols. After collection, the blood and tissue samples were stored at 4°C, were not exposed to room temperature for more than one hour and were processed within 8 hours post collection.

Peripheral blood (5-10 ml) was collected by venipuncture from each participant into EDTA-containing tubes. All blood samples were kept at 4˚C and centrifuged within 4 hours at 1200 x g for 10 minutes at 4˚C. Haemolytic samples, defined as pink-stained supernatant after centrifugation, were discarded. Thereafter a supernatant (plasma) was transferred into RNase-free eppendorf tubes in 0.5 ml aliquots and stored at -80°C until use. To eliminate any remaining cells, plasma was centrifuged again at 1200 x g for 10 minutes at 4˚C after thawing, immediately prior to RNA extraction. Each plasma aliquot was discarded after one freezing-thawing cycle.

Endometrial samples were collected under general anaesthesia prior to laparoscopy using a Pipelle suction curette (Pipelle de Cornier, Laboratoire C.C.D, Paris, France). All biopsies were immediately divided into 2 samples: 1. placed in RNA Later (Invitrogen, Carlsbad, CA) and stored at -80^{o} C for later RNA extraction; 2. immersed in 4% neutral-buffered Formalin for 24-72 hours, washed x3 in 75% Ethanol, placed in tissue cassettes and stored in 75% Ethanol up to 2-4 weeks until further processed and paraffin embedded. The samples were processed by using Processor LEICA TP 1020 following the manufacturer's protocol, which included dehydration in 2 changes of 95% Ethanol and 3 changes of 100% Ethanol for 30 minutes each and clearing with 3 consecutive baths of pre-cooled Xylene for 30 minutes each. Then samples

were embedded in Paraffin at 56°C and cooled at 4°C overnight. Formalin fixed paraffin embedded blocks were stored at room temperature in a standard block storage unit, and were used for histological evaluation of the tissue. Sectioning was carried out with a microtome into 5 µm thick sections, and after a few seconds of flotation in 40°C bath, the stretched section was picked up with a clean glass slide, dried at 65°C for 30 minutes and transferred to the histopathology unit at the Women's and Children's Hospital for staining and evaluation.

3.2 Optimization of RNA isolation from plasma

At the time of project commencement, published studies on circulating miRNAs were very few in numbers. Of these, each differed in the applied methodology. Moreover, the technical details and the quality assurance process were presented vaguely. Considering that we were one of the first to isolate miRNA from plasma, we put a substantial amount of work into the development of reliable reproducible techniques.

We chose to base the extraction process on the manufacturers' protocol for the Trisol LS reagent (Invitrogen, Life Technologies, Paisley, UK), which is a modified version of Trisol reagent, designed for liquid samples. For optimisation of the RNA isolation protocol, we performed a series of experiments, using a pooled sample collected from healthy volunteers. The factors assessed in the process of optimisation included: 1. determination of the optimal plasma to Trisol ratio, 2. identifying the optimal dilution of the plasma prior to extraction, 3. assessing the contribution of the small RNA carrier and 4. observations of the spike-in synthetic miRNAs as controls for extraction efficiency. Conventional spectrophotometric RNA quantification with a Nanodrop is not suitable for plasma samples, therefore the efficiency of the extraction process was validated by performance of qRT-PCR. qRT-PCR kinetics were assessed in the extracted samples, with inter and intra-experimental variability as well as inter-operator variability.

3.2.1 Plasma to Trisol ratio, plasma dilution, plasma volume

Given that lipids and protein in plasma are known to inhibit the PCR reaction, we decided to establish if a more diluted sample or a larger amount of denaturation solution (Trisol LS) resulted in better performance of qRT-PCR. We also determined the optimal starting volume of plasma that resulted in a good miRNA yield and was practically feasible.

First, we performed RNA extraction using 3 different starting volumes of plasma: 500 µl, 250 µl and 125 µl with a similar amount of Trisol LS in each experiment, testing different plasma

to Trisol LS ratios (1:3, 1:6, 1:12, respectively). Each extraction was performed in duplicate and tested using qRT-PCR with TaqMan miRNA specific primers to assess a relative expression of the synthetic miRNA cel-miR-54 and endogenous miRNA miR-126, miR-141, and let 7a. A constant amount (25 fmol) of cel-miR-54 was spiked-in to the plasma samples prior to extraction, whereas each of the endogenous miRNAs was demonstrated as present in human plasma samples. Neither synthetic nor endogenous miRNA were detected in samples extracted from the sample with 1:12 plasma to Trisol LS ratio. The RNA extracted from the sample with 1:6 plasma to Trisol LS ratio showed a higher Cq value (lower abundance) for both spike-in and endogenous miRNAs with higher variability between the extraction replicates for cel-miR-54, compared to the samples with 1:3 plasma to Trisol LS ratio (Figure 3.1). Thus, a 1:3 plasma to Trisol ratio was considered optimal; 500 µl of plasma was sufficient and at the same time permitted convenient handling throughout the procedure. Plasma dilution prior to extraction (1:1 with PBS buffer) was an unnecessary step and was associated with poorer results (Figure 3.2).

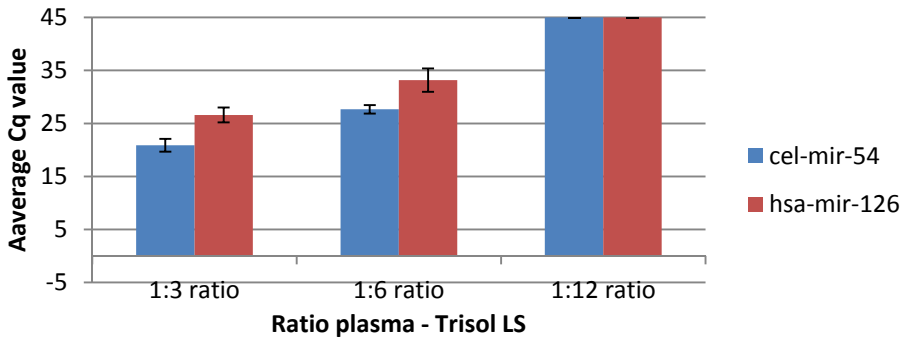


Figure 3.1 Optimisation of the RNA extraction – results of RT-PCR performed on the RNA sample extracted from different plasma – Trisol LS ratios.

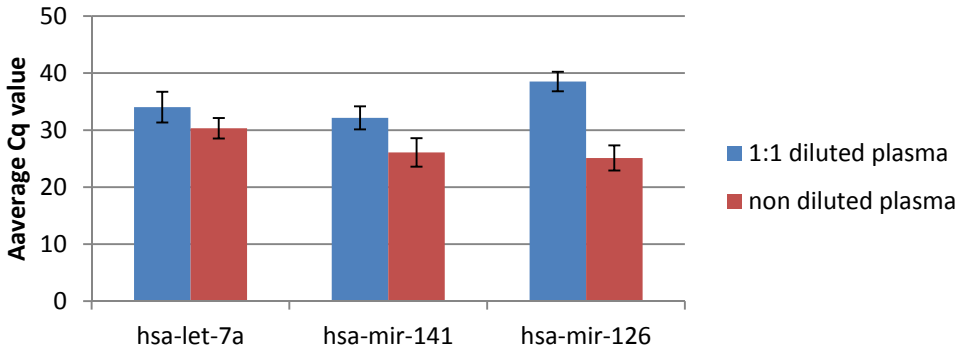


Figure 3.2 Optimisation of the RNA extraction – results of RT-PCR performed on the RNA samples extracted from diluted and from non-diluted plasma samples.

3.2.2 Spike- in synthetic miRNAs

Mitchell et al. (2008), introduced concept of spike-ing for qRT-PCR normalisation, suggesting a combination of 3 synthetic miRNAs from the C. Elegans species (cel-miR-39, cel-miR-54, and cel-miR-238) as a mixture of 25 fmol each in a 5 µl total volume of water (248). Determination of a normalized Cq value for the spiked-in miRNA, termed median-normalised Cq, was validated using the formula: Median-normalised Cq = Raw Cq value - [(spike-in average Cq value of the given sample) - (median spike-in Cq value)].

We assessed the use of the abovementioned spiked-in synthetic miRNAs as a putative internal control. Three synthetic miRNAs from C. Elegans (cel-miR-54, cel-miR-39 and cel-miR-238) were added to the plasma prior to RNA extraction in different quantities ranging from 6.25 to 50 fmol. This set of experiments aimed to determine the optimal amount of the spiked-in templates for our samples as well as to demonstrate that the technique was sensitive enough to correlate the Cq values with a changing amount of template in the sample. The Cq values for each of the miRNAs were within detectable range, showing a linear relationship with a strong correlation between the input amount and PCR measurements (Figure 3.3).

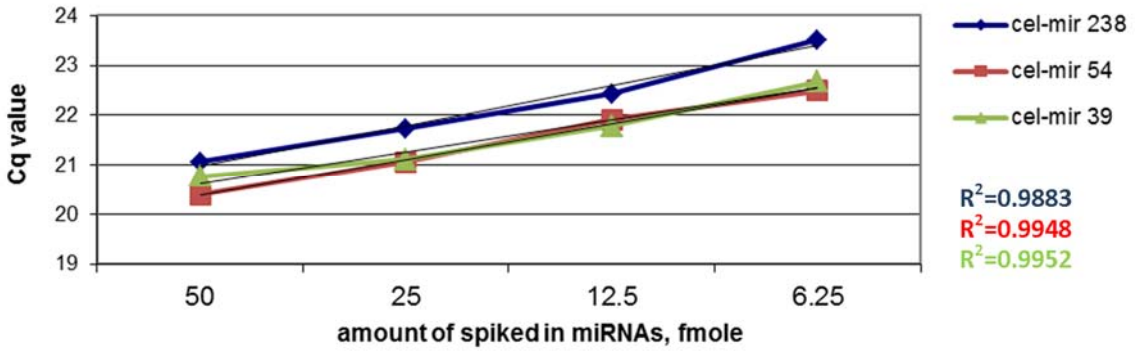


Figure 3.3 Correlation between the input amount of spike-in synthetic miRNAs and PCR results.

Even though we did not observe a direct relationship between the relative amount of input template and the recovery of the synthetic miRNAs measured as Cq values, (e.g. 8 times difference between the lowest and the highest spike-in amount corresponded to 4-5.7 post extraction difference or 2-2.5 Cq values), a clear difference between lower and higher amount of spiked-in templates was evident. The lack of an absolute correlation between input amount and Cq values could be explained either by partial loss of the input material during the extraction process or by a certain degradation level of the synthetic templates by endogenous RNases. Thus, we accepted that the spiked-in amount of 25 fmol, as suggested by others, resulted in reasonable Cq values and was included in the extraction protocol.

Considering the similarity in expression levels of the 3 synthetic miRNAs, we assessed the necessity of using all 3 vs. 1 selected template. The rationale behind this experiment was to try to reduce the number of spiked-in controls, and thus the subsequent RT-PCR reactions for their detection, making the quality control process less labour intensive. Overall, all the 3 spike-in synthetic miRNAs (cel-miR-39, cel-miR-54 and cel-miR-238) exhibited very similar patterns of expression across the samples, and any substantial sample-to-sample variability was reflected by very concordant Cq values for each of the 3 miRNAs (Figure 3.4).

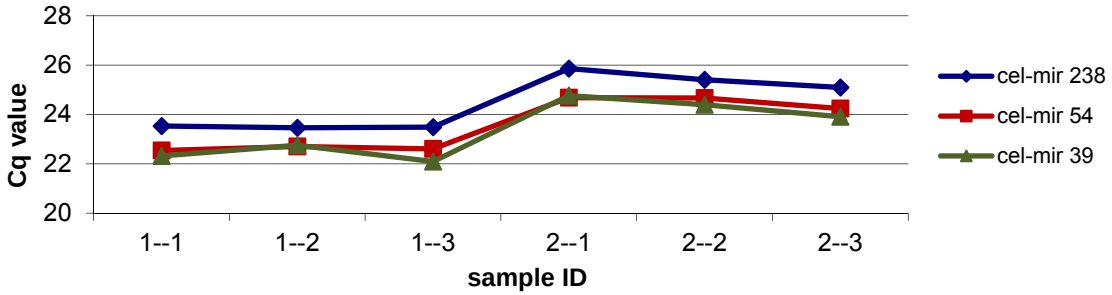


Figure 3.4 Relative measures of the 3 spiked-in synthetic templates across various samples.

The median-normalised Cq of endogenous miRNA, has-miR-16, showed similar values when normalised to each of the spike-in miRNAs or to their average, suggesting that each one of the synthetic templates performs similarly to the average of all 3 and can serve as an independent normaliser (Figure 3.5). We spiked-in one synthetic template, cel-miR-54 in the amount of 25 fmol in all experimental protocols.

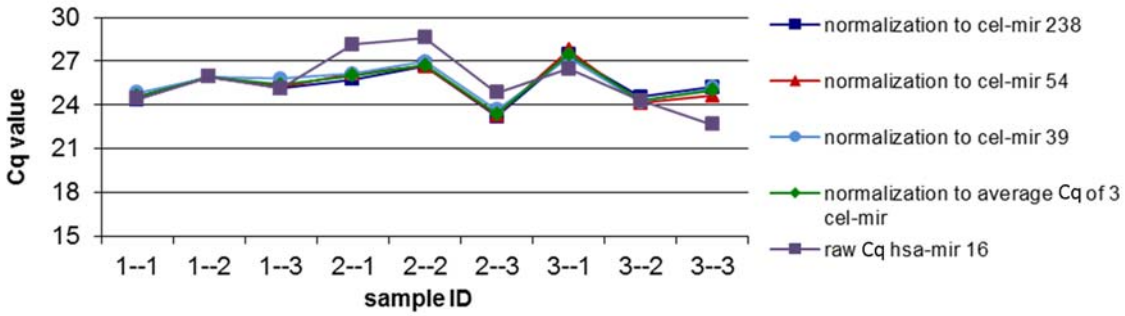


Figure 3.5 Raw and median-normalised Cq values of endogenous miRNA (hsa-miR-16) recovered from plasma. Normalisation was performed to the geometric average of the 3 miRNAs (as suggested by Mitchell) or separately to each spiked in miRNA.

Given the limited options to assess the quality of our samples, we used spike-ins as an important tool for assessing RNA extraction quality. The levels of cel-miR-54 were measured in each RNA sample and a SD was calculated for each PCR plate. Samples in which cel-miR-54 variance was above 2SD were considered as poorly recovered. RNA from these samples was then re-extracted from a fresh plasma aliquot. We also used spiked-in cel-miR-54 as a

reference to normalise the data across the samples in qRT-PCR experiments included in Chapter 4, as previously demonstrated by others (225, 248, 252, 258, 349). However, as we gained more experience, we ultimately decided not to use the normalisation method based on spike-in miRNAs in the next set of experiments. This decision was based on the fact that, unlike endogenous controls, a spiked-in template could not fully account for the technical variability associated with PCR performance, other than the extraction process. This for example includes lipids and proteins, which escaped denaturation and are known to inhibit the PCR reaction, as well as any difference in plasma input resulting from the pipetting errors. Our further experiments included endogenous controls that were specific to the experimental dataset. The putative endogenous controls were selected in the array experiment with the GeNorm algorithm and validated by qRT-PCR as stable, abundantly expressed miRNAs, as described in Chapter 7 and suggested in the recent methodological papers in the field (298, 304).

3.2.3 Inter- and intra-assay variability and use of carrier for small RNA

To test the reproducibility of the extraction method, we quantified the synthetic spike-in miRNA, cel-miR-54 and 2 endogenous miRNAs (has-miR-16 and has-miR-126) in RNA samples extracted on the different days in three replicates each from the same pooled sample. MiR-16 was suggested by previous studies to be a stable endogenous control that is abundant and consistent across the plasma samples (252), whereas miR-126 has been previously detected in plasma in various concentrations (245, 248). Initially, the variation in miRNA quantification between separate RNA isolations, as measured by the Cq values of spike-in miRNAs, was lower for the extractions performed on the same day, but was substantially higher between different days of extraction, whereas endogenous miRNAs had more substantial inter and intra-assay variability (Table 3-1). The addition of RNase-free small RNA carrier prior to the precipitation stage of extraction, improved RNA yield and reduced variability, similar to the reports of others (252, 269). The intra-experimental variability was reduced for both synthetic and endogenous miRNAs after plasma and Trisol LS were mixed by vortexing rather than by pipetting (data not shown). We implemented several steps to reduce intra-and inter-experimental variability, which included vortexing of samples after the addition of Trisol LS reagent and the addition of a small RNA carrier.

Table 3-1 Measures of inter and intra-experimental variability of miRNA extraction by the modified Trisol LS method used in our experiments[1]

	cel-miR-54		hsa-miR-126		hsa-miR-16	
	- small RNA carrier	+ small RNA carrier	- small RNA carrier	+ small RNA carrier	- small RNA carrier	+ small RNA carrier
Average Cq values	29.4	24.6	30.4	27.3	29.2	28.1
Intra-experimental variability	2.5 Cq	1.9 Cq	6.3 Cq	3.1 Cq	3.6 Cq	1.6 Cq
Inter-experimental variability	2.1 Cq	0.7 Cq	3.9 Cq	2.5 Cq	4.5 Cq	2.2 Cq

[1] The variability was assessed across the extraction replicates from the same pooled sample and measured by Cq values of one synthetic spike-in miRNA and 2 endogenous human miRNAs.

3.2.4 Intra-operator variability

We assessed the performance of the technique performed by 2 different operators. One operator was experienced in miRNA extraction from plasma samples (VN), whereas the second operator was familiar with the RNA extraction from tissues, but was extracting miRNAs from plasma for the first time. Three plasma samples were extracted in parallel by each investigator; extraction efficiency and yield was assessed by measuring cel-miR-54 in RT-PCR as well as by 2100 Bioanalyzer chip for small RNAs. Overall, the variability measured by qRT-PCR did not exceed 1 Cq. The Bioanalyzer readings differed more substantially, particularly in samples with very low and very high miRNA abundance, but in general were consistent between the operators (Figure 3.6). This suggested that the technique is reproducible and the inter-operator variability did not exceed the intra-experimental variability of the extraction performed in the same hands. Despite this, all the RNA samples analysed in the current work were extracted by the same operator (VN).

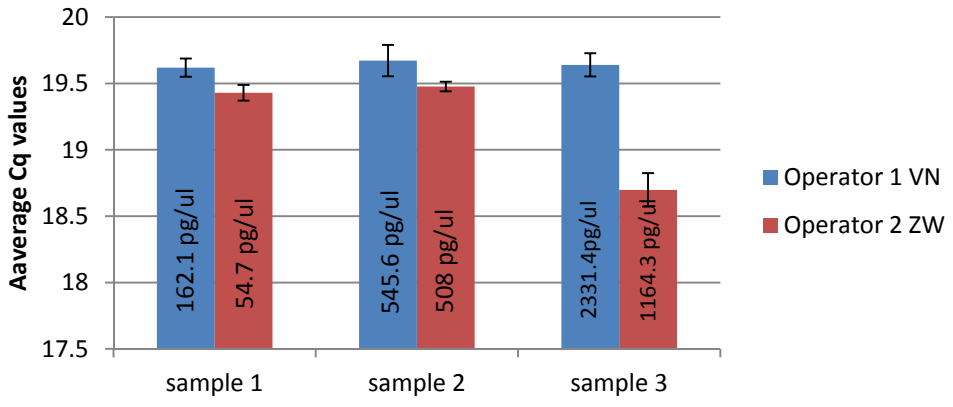


Figure 3.6 Assessment of the intra-operator variability of plasma RNA extraction. The plasma samples from 3 individuals were extracted by 2 operators (VN and ZW). The extraction quality and miRNA yield were assessed by measuring expression levels of cel-miR-54 with qRT-PCR (Cq values presented as bar charts) and by 2100 Agilent Bioanalyzer, chip for small RNAs. The concentration assessed with Bioanalyzer is presented as numbers above each corresponding bar.

3.2.5 Assessment of miRNA quality and quantity

In our hands, the spectrophotometric RNA quantification was not successful for plasma samples, presumably due to the low yield of recovered RNA that is below the limit of accurate quantification. We observed unusual spectra on Nanodrop, including very low 260/280 ratios, low 260/230 ratios and a very noticeable peak at 270nm were probably due to phenol contamination. However, testing with the TaqMan qRT-PCR indicated the presence of RNA and demonstrated that miRNA was of sufficient quantity and quality for quantification by qRT-PCR. A dilution of the sample (1:1 dilution of the input RNA and 1:10 dilution of the cDNA) was found to substantially minimise the effect of inhibitors on the reaction.

The quantities of miRNAs in the samples were assessed using Agilent 2100 Bioanalyzer (Agilent Technologies, Germany) chips for small RNAs. The miRNA across the plasma samples that were included in the further experiments accounted for 55%-92% of total small RNAs. The estimated median miRNA extracted with our modified Trisol LS protocol from 0.5 ml of plasma was 346.3 pg/µl, with values ranging from 154-796 pg/µl. This is equivalent to 7 – 16 ng per 1 ml of plasma and is consistent with the reports of others (245). RIN was not calculated with the Bioanalyzer due to low RNA concentration in plasma. For the same reason we did not perform RNA fractioning on agarose gels for RNA quality assessment.

We assessed RT-PCR kinetics as a sample quality measure, by using TaqMan miRNA assays. The RT reaction for both assays had reasonable efficiency and a dynamic range of 112%, detecting across 6 log10 dilution for cel-miR-54 and 110% with 5 log10 dilution for has-miR-126, respectively (Figure 3.7A).

Plotting of miR-126 Cq values normalized to cel-miR-54 (ΔCq) suggested that the accuracy of RT reaction was compromised with very high and very low RNA concentrations. Higher precision was demonstrated for cel-miR-54 with SD of PCR replicates = 0.7 R^2 = 0.99 compared to miR-126, with SD = 1.1 and R^2 = 0.74 (Figure 3.7B).

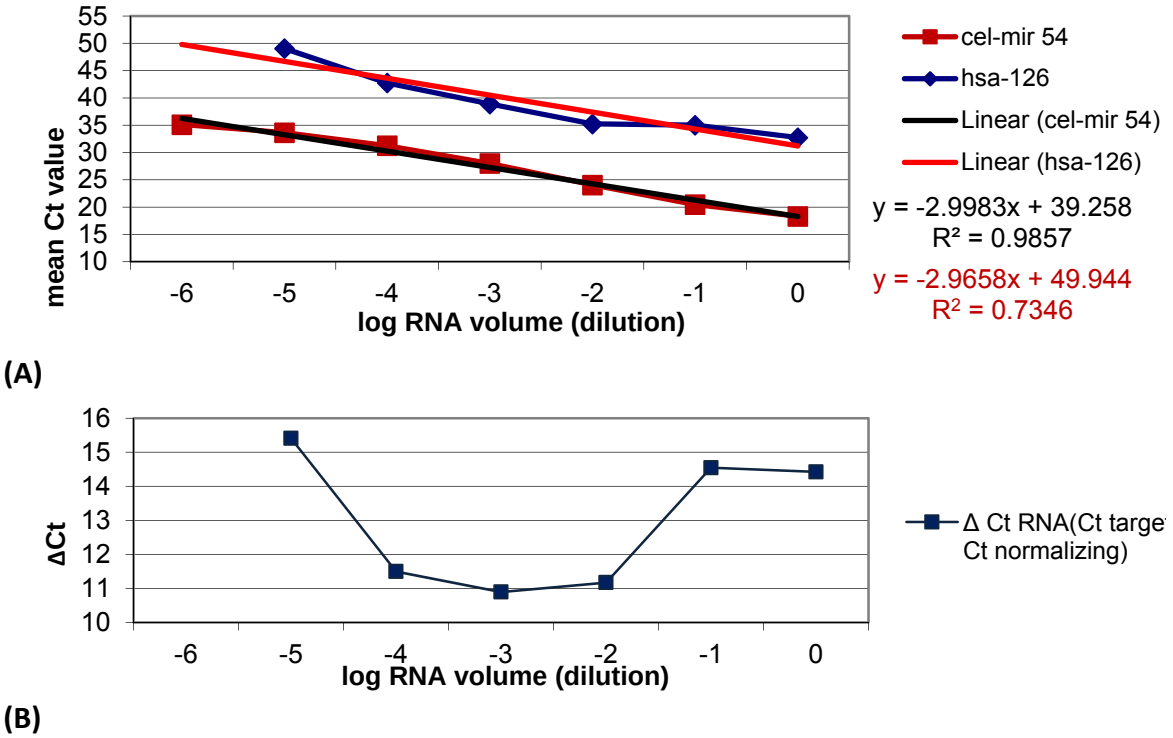


Figure 3.7 Kinetics of RT reaction for cel-miR-54 and hsa-miR-126 assays. The efficiency of the reaction was calculated by using a formula: PAE = $\log_2 AE = \log_2 10^{-1/slope}$.

The efficiency and dynamic range of the PCR reaction were within acceptable ranges (96% and 5 for cel-miR-54 assay; 112% and 4 for miR-126 assay, respectively (Figure 3.8)). The presence of a significant tailing effect at high concentrations for both assays suggested the need for dilution, and a 1:10 dilution of cDNA appeared to be sufficient. SD between PCR replicates appeared to be high at very low and very high concentrations of cDNA.

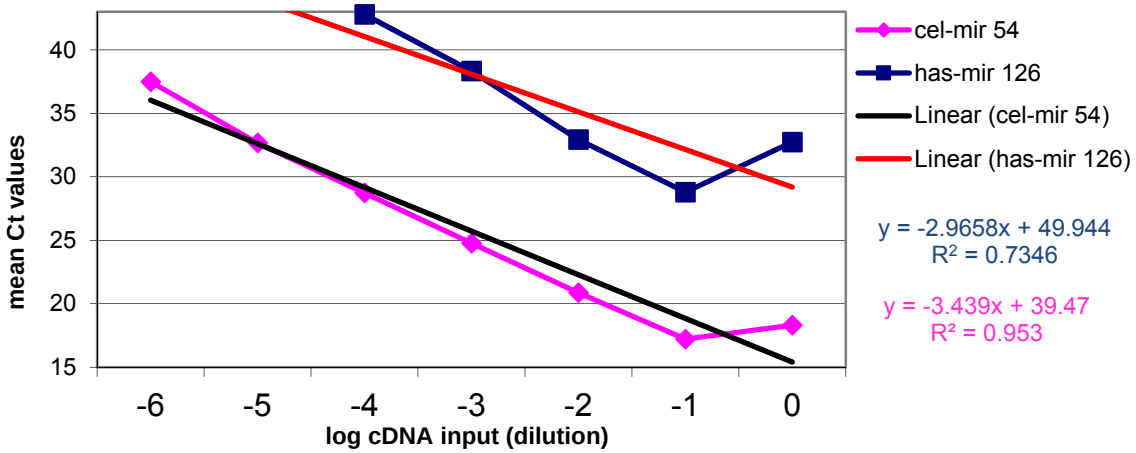


Figure 3.8 Kinetics of PCR reaction for cel-miR-54 and hsa-miR-126 assays. The efficiency of the reaction was calculated by using a formula: PAE = $\log_2 AE = \log_2 10^{-1/slope}$.

3.3 The protocol of RNA isolation from plasma, established in our laboratory

Total RNA was extracted from 0.5 ml of plasma using the Trisol LS reagent for liquid samples (Invitrogen, Carlsbad, CA) following the manufacturer's protocol with slight modifications as following:

1. Plasma was thawed on ice and Trisol LS reagent is added to plasma samples in volumetric ratios 1:3, the samples are mixed by vortex.
2. Then 25 fmol (5 µl of 5 nM stock solution) of synthetic C. Elegans miRNA cel-miR-54 was added to plasma samples (after addition of Trisol to avoid degradation by endogenous RNases).
3. After 5 min of incubation at room temperature, aqueous and organic phase separation was achieved by addition of molecular grade chloroform (400 µl per 1.5 µl of Trisol LS) followed by 15 seconds (sec) of shaking and 10 min of incubation at 4˚C.
4. The samples were then centrifuged at 12000 x g for 15 minutes at 4˚C, the upper aqueous phase was carefully transferred into a fresh tube and incubated for 10 minutes at room temperature with 2 volumes of isopropyl alcohol (Isopropanol, Sigma Aldridge) and with 75 µg of RNase-free carrier of small RNAs, glycoBlue (Invitrogen) to allow the RNA precipitation.
5. The mixture was then centrifuged at 12000 x g for 10 minutes at 4˚C, the supernatant was removed, while the pellet is washed with 4 volumes of 75% Ethanol by vortexing and centrifugation (7500 x g, 5 minutes, 4˚C).
6. Finally, the supernatant was discarded, and the RNA pellet is dried for 5-10 minutes at room temperature, dissolved in 10 µl of DEPC-treated water and stored at -80˚C.

Each plasma sample was extracted in duplicate, one replicate with and one replicate without the spike-in of a synthetic template.

The expression of spike-in cel-miR-54 was measured in triplicates by using qRT-PCR in 96 well plates, to control for the extraction efficiency and to identify PCR inhibitors in RNA samples. The expression levels of all samples were compared to the average expression of the plate. Samples with Cq values of > 2SD from the mean Cq of the plate were excluded and re-extracted. The yield of miRNA fraction was analysed in the second extraction replicate free of spike-in miRNAs by using Agilent 2100 Bioanalyzer (Agilent Technologies, Germany) chips for small RNAs.

3.4 MiRNA extraction from endometrial tissue

3.4.1 MiRNA extraction

Total RNA was extracted from 100 mg of frozen whole-tissue endometrial specimens homogenized in Trisol solution (Invitrogen Carlsbad, California) and subjected to DNase treatment according to the manufacturer's instructions. Briefly, endometrial tissue was thawed on ice, Trisol LS reagent was added to tissue samples in ratios 1 ml per 100 mg of tissue and homogenised with Ultra Turrax T8 Homogeniser. Trisol-homogenised samples were incubated for 5 minutes at room temperature and centrifuged at 12000 x g (13,000 rpm) for 10 minutes at 4°C. Supernatant containing RNA was transferred to a fresh 2 ml eppendorf tube, incubated at room temperature for 5 minutes to allow for dissociation of nucleoprotein complexes and vortexed. Aqueous and organic phase separation was achieved by addition of molecular grade chloroform (200 µl per 1.0 ml of Trisol LS) followed by 15 seconds of shaking and 3 minutes of incubation at room temperature. Then samples were centrifuged at 12 000 ×g for 15 minutes at 4°C, aqueous phase was transferred to a fresh 2 ml eppendorf tube and incubated for 10 minutes at room temperature with isopropyl alcohol (Isopropanol, Sigma Aldridge) (1 ml per 1 ml Trisol), mixed with a pipette and incubated for 10 min at room temperature to allow the RNA precipitation. The mixture was then centrifuged at 12000 x g for 10 minutes at 4˚C, the supernatant was removed, while the pellet was washed with 75% of ice cold Ethanol (1 ml per 1 ml Trisol) by vortexing and centrifugation (7500 x g, 5 minutes, 4˚C) to re-pellet RNA. Finally, the supernatant was discarded, and the RNA pellet was dried for 1-5 minutes at room temperature, dissolved in 50 µl of DEPC-treated water and incubated for 10 minutes on ice, followed by incubation at 55-60°C for 10-15 minutes with repeated pipetting to completely redissolve the RNA containing pellet. For the removal of contaminating DNA from RNA samples, DNase treatment was performed by using Ambion DNA-free™ kit (Invitrogen, Carlsbad, CA). 5 µg of RNA sample was diluted in 20 µl DEPC-treated H2O and incubated with 2 µl of 10 x DNase 1 buffer (or 0.1 x volume of RNA) and 1 µl of rDNase1 for 25 -30 minutes at 37°C. DNase inactivation reagent was resuspended by vortexing and gentle shaking and 5µl (or 0.1 volume, whichever is greatest) was added to RNA solution. The mixture was incubated at room temperature for 2 minutes by occasionally mixing the contents of the tube 2-3 times to redisperse DNase inactivation reagent and then centrifuged at 10 000 x g for 1.5 minutes at room temperature to pellet DNase inactivation reagent. The

supernatant containing total RNA was transferred to a fresh 1.5 ml Eppendorf tube. The RNA samples were stored at - 80°C until use.

3.4.2 MiRNA quantification and quality control

RNA concentration and purity was assessed using a Nanodrop spectrophotometer, ND-1000 (Thermo Fisher Scientific Inc., Waltham, MA, USA), by using 1 µl of RNA. Contamination from protein was assessed by ratio for sample absorbance at 260/280 (A260/A280), accepting A260/2A80 ratio of 2.0; contamination from organic compounds was assessed by ratio A260/A230, a ratio of 2.0 - 2.2 was consistent with the highest purity.

RNA integrity was assessed by using agarose gel electrophoresis, which uses the resolution of distinct 28s and 18s RNA bands to indicate the quality of the RNA. Briefly, 1% agarose gel was made by mixing of 0.6 gram agarose in 60 ml of 1 x TAE (Tris-acetate-EDTA) buffer (TAE is prepared from the mixture of 40 mM Tris-base, 1mM EDTA and 1.142 nl glacial acid). Agarose/TAE solution was heated for 1 min to dissolve agarose and cooled for 10 minutes. The 2 µl of 10mg/ml ethidium bromide (Sigma-Aldrich) was mixed into the solution. Gel was poured into a gel cassette, cooled for 10 minutes and placed into the gel tank covered with 1 x TAE buffer. Then 500 ng of RNA , diluted in 10 µl of DEPC water, was mixed with 2 µl of 6x gel loading buffer (the buffer is prepared by mixing of 30% glycerol [3ml 100% Glycerol] with 0.25% Bromophenol blue [25mg Bromophenol blue] and 10 ml of DEPC water). The RNA–loading buffer samples were loaded into gel, run at 80V for 40 minutes and read by using UV transilluminator. RNA quality was additionally assessed by using the 2100 Bioanalyzer (Agilent Technologies, Foster City, CA, USA), accepting RNA integrity number (RIN) > 7 as high quality RNA, RIN 4-7 as average quality and RIN < 4 as low quality RNA.

3.5 MiRNA profiling in plasma

The expression levels of circulating miRNAs were assessed by using 2 techniques: miRNA arrays and individual qRT-PCR assays. For miRNA expression screening in plasma we use TaqMan Low Density Human miRNA arrays (TLDA) v2.0 (Applied Biosystems, Foster City, CA, USA). The TLDA platform has been selected because it requires smaller amounts of RNA, less than 1 ng per experiment, which is about ~20 times less than what is required for other microarray hybridization platforms. TLDA consists of 2 pre-configured 384-well micro fluidic card sets (card A and card B) that permit screening of 674 human miRNAs which are mapped using miRNAs Sanger MiRBase v14.0 entry names. The names of the miRNAs of interest

presented in the results section of Chapter 4 and Chapter 6 were manually updated to the MiRBase v.18.0. Each array includes 1 negative control (ath-miR159a), unrelated to any mammalian species. Array card A contains generally highly characterized miRNAs, while card B contains many of the more recently discovered miRNAs. The majority of the targets on each array card are presented in single replicates, with the exception of the endogenous controls suggested by the manufacturer that are presented in several (2–4) technical replicates. Expression profile of miRNAs, selected on the basis of the miRNA array panel, was validated by qRT–PCR using single TaqMan miRNA assay kits (Applied Biosystems, Foster city, CA, USA).

In contrast to RNA extracted from tissue, only minute quantity of RNA are able to be extracted e from plasma and can not be measured reliably by UV-absorbance and the small chip Bioanalyzer. Therefore, the only available estimate of quantity in plasma miRNA work is volume. In our experiment fixed volumes rather than fixed amounts of RNA were used for the initial RT reaction in both array and individual qRT-PCR assays. The upload of the same volume into the RT reaction means that the RNA in the samples cannot be accurately quantified; however, this is considered a reasonable approach in the literature as all samples are handled in the same way (252, 262). We acknowledge that in order to develop a miRNA-based diagnostic blood test, better measures of quality and quantity for miRNAs retrieved from blood samples require development.

3.5.1 TaqMan Low density Human miRNA RT-PCR arrays

3.5.1.1 Procedure

All reagents were manufactured and supplied by Applied Biosystems, Foster City, CA, USA. CDNA was made by using Megaplex primer pools A and B followed by pre-amplification and PCR amplification steps. TaqMan Megaplex™ RT Primers are predefined pools of up to 381 primers for the one-tube megaplex reverse transcription of mature miRNA forms, which are complemented by the respective Megaplex TaqMan PCR probes. Preamplification of cDNA was undertaken using Megaplex™ PreAmp Primer pools consisting of forward primers specific for each of the miRNAs and a universal reverse primer. This step is recommended by the manufacturer both to generate enough miRNA cDNA template for the following PCR and to increase the sensitivity of the TaqMan miRNA profiling in samples with minute amounts of RNA. An initial RNA volume input of 3 µl per array was used. The total RNA plasma extract was reverse transcribed using the primers pool and reverse transcription kit in 7.5 µl containing 3 µl of RNA extract, 0.8 µl of sequence of Megaplex RT primers, 0.2 µl of 100 mM dNTPs, 1.5 µl

of Multiscribe Reverse Transcriptase (50 U µl -1), 0.8 µl of 10 x reverse transcription buffer, 0.9 µl of MgCl2, 0.1 µl of RNase inhibitor (20 U µl -1) and 0.2 µl of nuclease-free water. For cDNA synthesis the reaction mixtures were incubated for 40 cycles at 16°C for 2 minutes, at 42°C for 1 minute, at 50°C for 1 second, followed by incubation at 85°C for 5 minutes and then held at 4°C. Pre-amplification was performed in 25 µl reactions, including 2.5 µl of RT product (cDNA), 12.5 µl of pre-amplification master mix, 2.5 µl of Megaplex pre-amplification primers and 7.5 µl of nuclease-free water. The reaction mixes were incubated at 95°C for 10 minutes, at 55°C for 2 minutes, at 72°C for 2 minutes, followed by 12 cycles of 95°C for 12 seconds and 60°C for 4 minutes, then incubated at 99.9°C for 10 minutes and held at 4°C. The pre-amplified cDNA was diluted with 75 µl of nuclease-free water and stored at -20°C. The pre-amplified RT products were subsequently amplified with Megaplex human pool PCR primers; 9 µl of RT products were added to 441 µl nuclease free water and mixed with 450 µl TaqMan Universal Master Mix, no AmpErase UNG, then dispensed into the 384 wells by centrifugation and amplified using an ABI 7900HT Thermal Cycler (Cycling conditions: 95°C for 10 minutes, followed by 55 cycles of 95°C for 15 seconds and 60°C for 1 minute).

3.5.1.2 Quality control and downstream analysis

The results were reported as the Cq values, corresponding to the number of cycles required for FAM fluorescent signal to cross the threshold in RT-PCR. Raw Cq values were obtained using SDS v.2.3 and RQ Manager v.1.2 software (automatic baseline, threshold = 0.1). Cq values > 32 were considered to be below the detection limit and only miRNAs that showed a Cq ≤ 32 were considered as expressed. This threshold was set according to the manufacturer's recommendations for the TaqMan array analysis, suggesting that Cq values > 32 in multiplex reactions indicate target sequences of negligible abundance which cannot be precisely or reliably measured. All detectors with Cq > 32, or that failed amplification (marked as "Undetected" or "NA"), were assigned a value of 33. For the targets presented in more than 1 technical replicate, the Cq values were averaged if the replicates had SD < 0.5 across all samples. The technical replicates with SD ≥ 0.5 were removed from the analyses if there were more than 2 replicates, or analysed separately in case of duplicates.

In an attempt to identify the outliers amongst the biological replicates, visual inspection of the raw data per array plate was performed. We were cautious regarding the exclusion of the biological replicates and performed the separate analyses with and without exclusion of the poor performing "low abundance" plates. To assess the main source of variation in the

experiment, we applied unsupervised hierarchical clustering presented as a heat map. In order to evaluate if the data handling prior to analysis altered the conclusions, we performed separate analyses, including a broader range of the data by setting a detection limit cut-off at Cq > 35 and by including the "low call" detectors, i.e. the detectors present at least in 1 sample.

3.5.1.3 Identification of the "low call" detectors

In the study presented in Chapter 4, detectors not expressed in above 50% of samples in each subgroup for each card were considered as "low call" detectors and were removed from further consideration. In the study presented in Chapter 7, any detectors expressed in less than 70% of samples in each subgroup for each card were considered "low call" detectors and were removed (see below in section entitled "Differences in analytical approach to TLDA data between 2 sets of experiments (Chapter 4 and Chapter 7)").

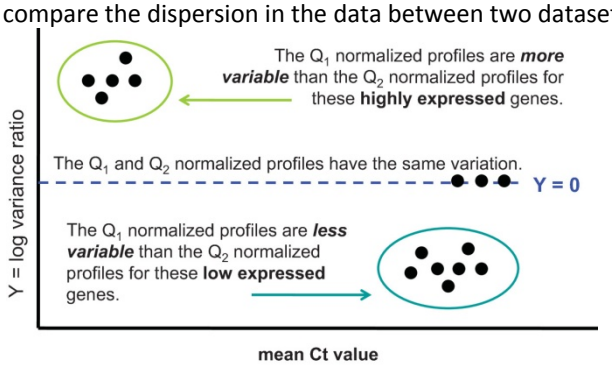
3.5.1.4 Identification of normalizing method

As no consensus on normalisation has been defined for high throughput miRNA array experiments and given that different ways of normalising data can potentially influence the results, we investigated several normalisation approaches incorporated in qPCRNorm package from the Bioconductor: 1. quantile normalisation, 2. rank invariant normalisation and 3. normalisation by the reference (house-keeping) miRNAs detected with the GeNorm algorithm (305). We compared the normalisation methods by using approaches presented in Table 3-2. For the GeNorm-based method, we evaluated the putative reference miRNAs amongst miRNAs that were readily expressed and present in all samples using GeNorm algorithm, separately for each array card (A and B). In the GeNorm program, an average expression stability value M was calculated for all candidate genes and those genes with the lowest M values were considered to be the most stable. The optimal number of endogenous controls required for accurate normalization was obtained by calculating the pair-wise variation values (V(n/n+1)) between each combination of sequential normalization factors, using the suggested cut-off value of 0.15, below which the inclusion of any additional controls does not result in a significant improvement of the precision (305).

Relative expression was calculated by the equation 2-quantile normalised Cq. FC was calculated by ΔΔCt with the formula $FC = 2^{-(\text{quantile normalised Cq in endometriosis} - \text{quantile normalised Cq in controls})}$. The FC represents the least FC, given all Cq values above 32 were not precisely

quantified, and thus the actual magnitude of the differential expression was under-estimated. The normalised data was analysed using the LIMMA.

Table 3-2 Measures to identify the more suitable normalisation approach; assessment a degree of variability of the raw and normalised data

Comparison measures	Description
Boxplot	Presents a degree of dispersion and skewness in the data, via presenting a sample minimal and maximal observations, median as well as lower and upper quartile
Histogram	Represents the density of data, by plotting tabular frequencies vs. signal intensities (Ct values)
Coefficient of variation (CV)	Is defined as a ration of the SD to the mean, generally used to compare the dispersion in the data between two datasets
Visual pair wise comparisons between the normalisation algorithms	 The log_2-ratio of variances of 2 normalisation algorithms Q1 and Q2 obtained for each target Y = log_2 [Q1-normalized: Q2-normalized] is plotted on the y-axis against its expression (mean Ct value) on the x-axis. The regions where the data points fall in the graph give us an indication of which normalization algorithm produces noisier data and whether there is a differential bias in expression for genes most affected by this noise (301).

3.5.1.5 Differences in analytical approach to TLDA data between 2 sets of experiments (Chapter 4 and Chapter 7)

The array experiment on the same set of samples was used as a basic screening tool in 2 included studies (Chapter 4 and Chapter 7). Considering different aims in each of these studies, we undertook different approaches to the experimental array data analyses. The study presented in Chapter 4 was a basic research study that aimed to characterise a miRNA profile in women with and without endometriosis across the different stages of the menstrual cycle. Therefore, miRNAs that were present in above 50% of the samples were selected for the analysis and analysis was performed separately at each menstrual cycle stage. The study presented in Chapter 7 aimed to identify diagnostic markers for endometriosis. Several modifications to the data analysis were performed to select a broad range of the putative biomarkers suitable for further validation. Firstly, any detectors expressed in less than 70% of samples in each subgroup for each card were considered “low call” detectors and were

removed. We undertook this approach assuming that a reliable biomarker should be expressed in the majority of the samples. Secondly, we increased the power of the analysis by combining all 3 time points of the cycle and compared all endometriosis to all control samples unrelated to the cycle stage. This analysis was only able to be performed because we did not demonstrate any significant differences in miRNA expression across the menstrual cycle (Chapter 4).

3.5.2 Singleplex qRT–PCR

3.5.2.1 Procedure

The expression profile of miRNAs of interest, selected on the basis of the miRNA array panel, was validated by qRT–PCR using single TaqMan miRNA assay kits (Applied Biosystems, Foster city, CA, USA) according to the manufacturer's instructions. Briefly, RNA samples were reverse transcribed using assay-specific primers and a reverse transcription kit in 7.5 µl containing 2.5 µl of RNA extract, 0.15 µl of 100 mM dNTPs, 1 µl of Multiscribe Reverse Transcriptase (50 U µl -1), 1.5 µl of 10 x reverse transcription buffer, 0.19 µl of RNase inhibitor (20 U µl -1), 3 µl of sequence-specific miRNA primers and 4.16 µl of nuclease-free water. For synthesis of cDNA the reaction mixtures were then incubated for 40 cycles at 16°C for 2 minutes, at 42°C for 1 minute, at 50°C for 1 second, followed by incubation at 85°C for 5 minutes and then held at 4°C. PCR amplification was carried out by using miRNA-specific primers in a final volume of 10 µl, containing 0.665 µl of the cDNA, 0.5 µl of primer, 5 µl of Master Mix with no AmpErase UNG and 3.8 µl of nuclease-free water. Three RT replicates per sample were used for amplification on an ABI 7900HT Thermal Cycler under the cycling conditions that similar to the TLDA experiments. No Reverse Transcriptase (NRT) and NTC controls were run simultaneously in duplicates.

3.5.2.2 Analysis of the data obtained in the singleplex qRT-PCR experiments in the study presented in Chapter 4

The Cq values were calculated using the SDS v2.3 software (Applied Biosystems) (automatic baseline, threshold = 0.2). A Cq value > 35 was taken as an indication of absence of the target miRNA (350). We normalised the data across the samples to a spiked control [cel-miR-54] using the median normalisation procedure, as shown previously (225, 248, 252, 258, 349). Due to the lack of agreement as to which endogenous controls are superior for the validation RT-PCR experiments, and because the set of reference controls identified by GeNorm showed

considerable variability in our array experiment, we assumed that adding the same amount of spiked-in RNA with an equal volume of plasma would provide a stable reference control. MiRNA-quantification results were expressed as median normalised Cq values: Normalized Cq value for the miRNA in the sample = Raw Cq value - [(Spike-in Cq value of the given sample) - (Median Spike-in Cq value in all tested samples)]. The molar concentrations of the spike-in were derived empirically to produce Cq values comparable to those of moderately abundant miRNAs measured in blood from human plasma (248). As shown in our preliminary experiments and supported by others (258), the imprecision in miRNA results is not altered by the number of spiked-in miRNAs.

3.5.2.3 Analysis of the data obtained in the singleplex qRT-PCR experiments in the study presented in Chapter 7

The expression levels of miRNAs were calculated using corrected for efficiency comparative ΔCq method (334). Following an export of the raw data and after removal of the Cq values > 35, Cq values were corrected for amplification efficiency of each individual miRNA primer, established using the standard curve method. Standard curves were generated for each of the tested assays based on 5 ten-fold serial dilutions of the input sample. The input product for the standard curves included pooled RT product of the tested samples. Amplification efficiency was calculated based on the slope of Cq plotted against the logarithm of the amount of input miRNAs using formula: and PAE = $\log_2 10^{-1/slope}$. Corrected Cq was calculated using formula Cq*$\log base_2$ ($10^{-1/slope}$), corresponding to what the averaged Cq values would have been had they amplified at 100% Efficiency.

We questioned the spiked-in synthetic miRNAs as internal controls. While able to correct for the RNA extraction quality, spiked-in synthetic templates are not able to control for the variations in starting sample quantity and for variable PCR efficiency due to numerous inhibitors contained in plasma that contaminate the purified RNA and interfere with subsequent enzymatic reactions. Therefore, we assumed that reliable endogenous controls should be inherently present in the plasma samples, subjected to identical conditions (purification method, storage, etc.) as target molecules and should undergo the same detection process on the same reaction plate. This allows controlling for as many technical variables as possible and makes all subsequent calculations independent of from RNA input of the samples.

The levels of miRNAs were normalized to a normalization factor [geometric averaging of miRNAs selected as a reference], so that all subsequent calculations were independent from RNA input of the samples. The ΔCq was calculated by subtracting the Cq values of normaliser from the Cq values of the miRNA of interest. The ΔΔCq was then calculated by subtracting ΔCq of the controls (disease-free) from the ΔCq of endometriosis samples. Expression levels were calculated by the equation $2^{-\Delta Cq} = 2^{-[Cq\ target-Cq\ normaliser]}$. Fold changes were calculated as $2^{-\Delta\Delta Cq} = 2^{-[\Delta Cq\ endometriosis-\Delta Cq\ controls]}$. All samples were processed under the same conditions and run by the single operator blinded to the surgical outcomes. The expression levels of the investigated miRNAs between the groups were compared using non-parametric statistics considering the number of the samples in each group and lack of the normal distribution of the data.

3.5.2.3.1 Selection and validation of the endogenous controls for quantification of plasma miRNAs

A systematic approach was undertaken to identify suitable endogenous controls. First, we identified a set of putative normalisers in the PCR array experiment via GeNorm algorithm. Next, the selected endogenous controls were subjected to qRT-PCR assay using 20 samples. An ideal endogenous control generally demonstrates expression that is relatively constant and highly abundant across the samples. Additionally, the reference miRNAs have to belong to different families to minimize the risk of co-regulation. Unlike profiling studies on miRNA-abundant tissues, where the same amount of miRNA is used for each experiment, the experiments on circulating miRNAs are based on the volume input, which can be associated with up to 4-5 times difference in miRNA amount. Endogenous controls would be expected to control for this level of disparity, however this difference could interfere with level of the controls themselves. Although endogenous controls were expected to demonstrate variability, the expression difference between the study groups should be minimal. Given that the megaplex primers were separate for each card and the PCR reactions in each pool were run on separate plates, we aimed to select separate normalisers for the assays in pool A and B. The assessment of validity of the endogenous controls included the following:

1 – each of the selected candidate controls should demonstrate negative NTC signals; 2 - the serial dilutions of the purified RNA samples should reveal excellent assay linearity; 3 - each of the selected candidate endogenous controls should be readily detected across the samples; 4 – the candidate controls should have small variation across the samples; 5 – the putative

endogenous controls should not exhibit expression differences between the groups (endometriosis vs. controls).

3.6 MiRNA profiling in endometrial tissue

3.6.1 MiRNA microarray and analysis

RNA samples were hybridized to the GeneChip miRNA 2.0 Array (Affymetrix, Inc, Santa Clara, California), with 15,644 miRNA probe-sets for mature miRNAs which have 100% coverage of miRBase v15 (135 organisms) The samples prepared for microarray analysis according to the Affymetrix protocol, at the Adelaide Microarray Centre, Hanson Institute, Institute of Medical and Veterinary Science (IMVS) Core Facility. Briefly, 500 ng of total RNA was tailed and ligated to FlashTag-Biotin-HSR using the Genisphere FlashTag Biotin HSR RNA Labelling Kit for Affymetrix GeneChip miRNA Arrays (Affymetrix). For poly-A tailing, the volume of RNA was adjusted to 8 µl with Nuclease-Free Water, transferred to ice and spiked with 2 µl RNA Spike Control Oligos. The 10 µl RNA/Spike Control Oligos was mixed with 5 µl of master mix for a total volume of 15 µl. The master mix included 1.5 µl 10X Reaction Buffer, 1.5 µl 25 mM $MnCl_2$, 1.0 µl diluted ATP Mix (the ATP mix was diluted 1:2000 in 1mM Tris, the dilution factor was calculated according to the formula 1000 ÷ µg input total RNA), 1.0µl PAP Enzyme. The mixture was gently mixed and incubated in a 37°C heat block for 15 minutes. For flash tag Biotin HSR ligation, 15 µl of tailed RNA was placed on ice, mixed with the 4 µl 5X FlashTag Biotin HSR Ligation Mix and 2 µl of T4 DNA Ligase followed by incubation at 25°C for 30 minutes. The reaction was stopped by adding 2.5 µl HSR Stop Solution. The ligated RNA was then hybridised to Affymetrix miRNA v2.0 arrays. The hybridisation step included mixture of 21.5 µl of the biotin-labelled sample with 50 µl 2X Hybridization Mix (from GeneChip Hyb, Wash and Stain Kit), 15 µl 27.5% Formamide , 10 µl DMSO (from GeneChip Hyb, Wash and Stain Kit), 5 µl 20X Eukaryotic Hybridization Controls (from GeneChip Eukaryotic Hyb Control Kit), 1.7 µl Control Oligonucleotide B2, 3 nM (from GeneChip Eukaryotic Hyb Control Kit) followed by incubation at 99°C for 5 minutes, then at 45°C for 5 minutes and injection of 100 µl of the reaction mix into an array. The array trays were then loaded into the hybridization oven and incubated at 48°C and 60 rpm for 16 hours.

The data were scanned according to the protocol described in Assay Manual from Affymetrix using an HR3000 scanner. The data generated by the Affymetrix GeneChip Operating Software analysis of the scanned array images were imported into Partek Genomics Suite software

(Partek, Inc, St. Louis, Missouri, US). The data files containing the probe level intensities were processed using the Partek Robust Multi-Chip Average analysis adjusted for the GC content of probe sequence (GCRMA), which included the background adjustment, normalization, and $\log_2$ transformation of perfect match values. The normalized data were used in pairwise comparisons of endometriosis and symptomatic control groups, endometriosis and asymptomatic controls as well as endometriosis and all controls. The miRNAs that were differentially expressed were identified using the one-way ANOVA as implemented in the Partek tool. The data presented as intensity values for each probe cell (cel.file) was also analysed by using the Affy package, Bioconductor. The data was background corrected, quantile normalised, summarised using the standard RMA method and analysed by using LIMMA.

3.7 Statistical analysis

3.7.1 Demographic characteristics

Values are expressed as mean ± SD or medians (range). The patients' baseline demographic and clinical characteristics were compared using Student's t-test or Mann-Whitney U test when appropriate for continuous variables and Fisher's exact test for categorical variables. The Shapiro-Wilk test was used to establish whether the continuous data followed the normal distribution.

The Spearman rank order correlation test was used to examine correlation relationships between the variables. Correlation coefficients (r_s) with a magnitude of 0.9 - 1.0 indicate very highly correlated variables, 0.7 - 0.9 - highly correlated variables, 0.5 - 0.7 - moderately correlated variables and 0.3 - 0.5 indicate low correlation. Little if any correlation between the variables was indicated by correlation coefficients of < 0.3.

3.7.2 TLDA miRNA experiment in plasma

The statistical analysis of the microarray data has been performed in consultation with Associate Professor Cristin Print, the head of the Bioinformatics Institute, Auckland University, Auckland, NZ. Comparative analyses of the array data were made applying empirical Bayesian moderation of standard errors and linear models for microarray data [LIMMA, Bioconductor tools in R (version 2.13.0)]. In the experiment presented in Chapter 4, the comparison of miRNA levels between three time points of menstrual cycle: early proliferative, late proliferative and mid-luteal and was made separately within each group (women with

endometriosis and healthy controls) using a nested F-test approach. If the overall F-statistic is significant, then the function assesses which t-statistic is most likely to have contributed to this result. The comparisons between endometriosis and control groups were made separately for each stage of the cycle using moderated t statistics. MiRNAs with p-values < 0.01 and FC > 1.5 were considered as differentially expressed. In parallel, a B–H method for multiple-testing correction was applied to keep the FDR under 5%. Considering the exploratory "hypothesis generating" nature of the study and in order to avoid too many type 2 errors, we did not apply the correction for multiple testing as a mandatory criterion for the significance of differential expression (322). The probability level for trend for statistical significance was determined as $0.01 < p < 0.05$.In the experiment presented in Chapter 7, the analysis was performed between the endometriosis and control groups irrespective of the menstrual cycle stage. In this study, only miRNAs that significantly differed between the groups and passed a test for multiple test correction, keeping FDR < 5%, were considered for the subsequent validation.

The power calculation to determine the sample size for the validation RT-PCR experiment (presented in Chapter 7) was not calculated. This is because it appears challenging to establish the two important components of the power calculations in context of the microarray experiment, i.e. the variance of the relative expression levels across the arrays and the magnitude of the effect of interest for individual miRNAs (351). Therefore, we included the maximal available number of samples in the validation experiment. Due to relatively small sample size, no subgroup analyses were performed to differentiate between the different types of endometriosis, i.e. ovarian vs. peritoneal or between the endometriosis sufferers with and without infertility for the participants included in the array experiment (presented in Chapter 4).

3.7.3 Affymetrix hybridisation miRNA microarray in endometrial tissue

Two computational methods (ANOVA and LIMMA) were used to estimate differential expression of miRNAs between the groups and miRNAs that were common to both methods were selected as differentially expressed. An estimate of the associated FDR was computed per miRNA using the method of B-H in both analyses, aiming to keep the FDR < 5%. Similarly to stated in Chapter 4, we did not apply the correction for multiple testing as a mandatory criterion for the significance of differential expression (322) in order to avoid premature

elimination of potentially useful observations. However, we applied stringent selection criteria for the miRNAs that did not pass the correction for multiple testing. The miRNA transcripts were considered to be significantly differentially expressed if they obtained p-value < 0.01 and were significant according to both methods. The probability level for trend for statistical significance was determined as $0.01 < p < 0.05$.

3.7.4 Heat maps and Venn diagrams

Heat maps were used to look for similarities between miRNA levels within each sample and were represented by the row Z-score, which was calculated by subtracting the mean of the row from every value and then dividing the resultant values by the SD of the row Z-score. A heatmap graphically depicts the measured intensity values of the miRNAs with the dendrogram illustrating relationships between the specimens and was generated by using gplots package in R, Bioconductor (352). Venn diagrams for comparing between the datasets were constructed by using Venny, the online interactive tool (http://bioinfogp.cnb.csic.es/tools/venny/index.html) (353).

3.7.5 qRT-PCR experiment

Expression levels of plasma miRNAs in single RT-PCR were compared using the Mann-Whitney U test or the Kruskal-Wallis test where appropriate. The post hoc analyses were done with Mann-Whitney U test using Bonferroni adjusted p-values. All p-values were exact two-sided and $p < 0.05$ was considered the threshold for statistically significant differences. P-values of 0.05 – 0.099 were considered to be suggestive of a trend of statistical significance. To assess if any of the demographic or clinical parameters that significantly varied between the groups had confounding effect, a correlation between miRNA expression (for each of the tested miRNAs) and a parameter of interest was assessed separately in each group.

3.7.6 Subgroup analyses (study presented in Chapter 7)

To examine whether the plasma levels of the investigated miRNAs may fluctuate under different conditions, the subgroup analyses were performed on a cohort of the participants stratified based on the factor of interest. The impact of the severity of endometriosis on miRNA expression was tested in 2 subgroups, minimal – mild disease (rASRM stage I-II) and moderate – severe disease (rASRM stage III-IV), providing there were no significant expression differences between the pooled together rASRM stages. To examine whether the plasma levels of the investigated miRNAs vary across a menstrual cycle, the participants were

stratified by the 4 stages of the cycle: menstrual or early proliferative, corresponding to day 1-4 of the cycle, proliferative, consistent with day 5-12, peri-ovulatory, day 13-14 and luteal, day 16-26. These subgroup analyses were limited only to women with regular menstrual cycles and not on contraceptive hormonal medications; menstrual cycle stage was determined based on verbal confirmation of a day of the cycle with consideration of the cycle length. Additional discriminative factors of interest for the subgroup analyses included: 1. endometrioma, 2. recurrent endometriosis, 3. hormonal treatment. When meaningful subgroup analysis was not possible due to limited sample size in one of the subgroups ($n < 5$), analysis was performed after exclusion of the subgroup of interest as an indirect way to assess the impact of an excluded parameter on the miRNA expression in the cohort. This was applied to 1. pelvic pathologies and general medical conditions on miRNA expression, which included a list of low prevalent conditions; 2. previous endometriosis in currently disease-free women; 3. hormonal treatment.

3.7.7 Diagnostic performance of plasma miRNAs

Receiver-operating characteristics (ROC) curves were established to evaluate the diagnostic value of plasma miRNAs for differentiating between endometriosis and controls for each individual miRNA and by the combinations of the selected miRNAs. The area under the ROC curve (AUC) was used as a measurement of the level of separation of settings. Univariate logistic regression analyses were taken to select the variables for the multivariable analyses. A multivariate logistic regression model was established. The Hosmer-Lemeshow test was used to test for goodness of fit in the logistic regression model. The sensitivity and the specificity were calculated according to the standard formulas. Statistical analyses were performed using R v2.13 and SPSS 18.0 software (SPSS, Inc., Chicago, IL).

3.8 Identification of predicted miRNA targets and in silico functional analysis

To identify targets of dysregulated miRNAs, two different methods were applied: the IPA miRNA Target filter, Ingenuity Systems Pathway Analysis software (IPA last updated 08.05.2012, Ingenuity Systems, Inc., Redwood, CA) and MirTarget2 tool incorporated miRDB database (www.miRDB.org), version 4.0, released January 2012, last updated 03.04. 2012. The miRNA Target Filter in IPA identified miRNA–mRNA interactions from TargetScan algorithm (TargetScan Human 5.1), TarBase v.5c, miRecords, and Ingenuity Knowledge database (IKB). TargetScan identifies predicted mRNA targets for miRNAs by searching for the presence of a

conserved seed match (i.e., perfect Watson-Crick complementarity [A:U or G:C matches] between nucleotides 2-7 of the miRNA) and a 6 nucleotide section of the 3 UTR of the mRNA), and either a conserved anchoring A (which is an A nucleotide on the 3' UTR just downstream of the seed match) or a conserved m8-t8 match (which is an A:U or G:C match between the eighth nucleotide of the miRNA and the corresponding position on the 3' UTR), or both. These relationships are binned into high and moderate predicted confidence. High confidence is assigned either when the relationship is between a conserved or highly conserved miRNA and at least one conserved site on the targeted sequence, or the total context score, as defined by TargetScan, is -0.4 or less. Moderate confidence is assigned if the total context score, as defined by TargetScan, is -0.2 or less. TarBase, miRecords and IKB are the manually curated collections that identify experimentally demonstrated miRNA/mRNA relationships from thousands of miRNA-related findings from the peer-reviewed literature. Only targets that were experimentally validated or predicted with the high confidence were selected.

MiRDB is an online database for miRNA target prediction and a functional annotation that implicates a SVM learning machine based bioinformatics tool MirTarget2 for target prediction. The computational target prediction algorithm assigns a prediction score of 50 – 100 to each target, which directly correlates with the prediction confidence. Only targets with a prediction score of > 80 were included in functional analysis. This additional target prediction algorithm (MirTarget2 tool) was implemented to ensure the completeness of the predicted mRNA target lists incorporated in the IPA and both target datasets were merged.

In silico functional analysis of the identified targets was performed by using IPA core analysis and IPA Path Explorer in order to identify the signalling pathways, molecular networks and biological processes that are most significantly perturbed in the dataset of interest. IPA uses information retrieved from the IKB, including manually curated collections that identify experimentally demonstrated miRNA/mRNA relationships from thousands of the peer-reviewed papers.

The top biological functions and canonical pathways identified in IPA were selected based on significance for the likelihood of association between the selected molecules and a given process. The p-value was calculated using the right-tailed Fisher's exact test by considering the number of functional analysis molecules that participate in that function and the total number of molecules that are known to be associated with that function in the IKB.

The molecular networks were developed from information contained in the application and were ranked according to their degree of relevance to the network eligible molecules in the dataset. The ranking score represents a numerical value that takes into account the number of network eligible molecules in the network, the total number of molecules analysed and the total number of molecules in the IKB that could potentially be included in networks. The network score is based on the hypergeometric distribution and is calculated with the right-tailed Fisher's exact test to estimate the approximate "fit" between each network and the network eligible molecules in a dataset, corresponding to the negative log of this p-value.

Additional analysis was restricted to the predicted targets in current study that intersected with the 4 previously published datasets of mRNAs identified in endometriosis. Four gene micro array datasets were downloaded from the GEO repository http://www.ncbi.nlm.nih.gov/projects/geo; with accession numbers GSE23339 (205) and GSE7305 (84) for endometrioma and eutopic control endometrium comparisons as well as GSE11691(85) and GSE5108(83) for ectopic and eutopic endometrium comparisons. There were 116 up-regulated and 72 down-regulated genes that intersected these 4 datasets (205) and these miRNAs were included in an *in silico* functional IPA analysis.

3.9 Strategy for conducting the diagnostic accuracy study

The studies of diagnostic accuracy compare results from one or more tests to the reference standard on the same subjects and are a vital step in the evaluation of any new diagnostic technology (354). Exaggerated results from poorly designed studies can trigger premature adoption of diagnostic tests and can mislead the clinical and research community to incorrect decisions about applicability of the test. The methodological quality of many of the reported studies was found to be less than optimal and information on key elements of design, conduct and analysis of diagnostic studies was often not reported (355). We revised the criteria for conducting and reporting of the diagnostic accuracy studies based on the STARD (Standards for Reporting of Diagnostic Accuracy) statement (356) and the QUADAS tool (the quality assessment of studies of diagnostic accuracy included in systematic reviews) (357-360). The results of this work were incorporated into our published protocol for a systematic review of the literature on the non-invasive tests for endometriosis for the Cochrane collaboration. We also implemented this methodology in our prospective diagnostic accuracy study on plasma miRNAs as diagnostic markers of endometriosis, presented in Chapter 7.

Both STARD and QUADAS provide the checklists and flow diagrams that should be followed for optimal study design, which aims at an unbiased diagnostic study. The flow chart for the STARD checklist is presented in Figure 3.9. This flow chart outlines the method of patient recruitment, the order of test execution, and the selection of an appropriate reference standard test. Each flow phase includes a section for an inconclusive test finding and provides a venue by which this can be identified.

The QUADAS tool, presented in Table 3-3, provides an organised format, which includes 14 components addressing a process of the patients' recruitment and allocation, execution of an index test and a reference test and approach to the data.

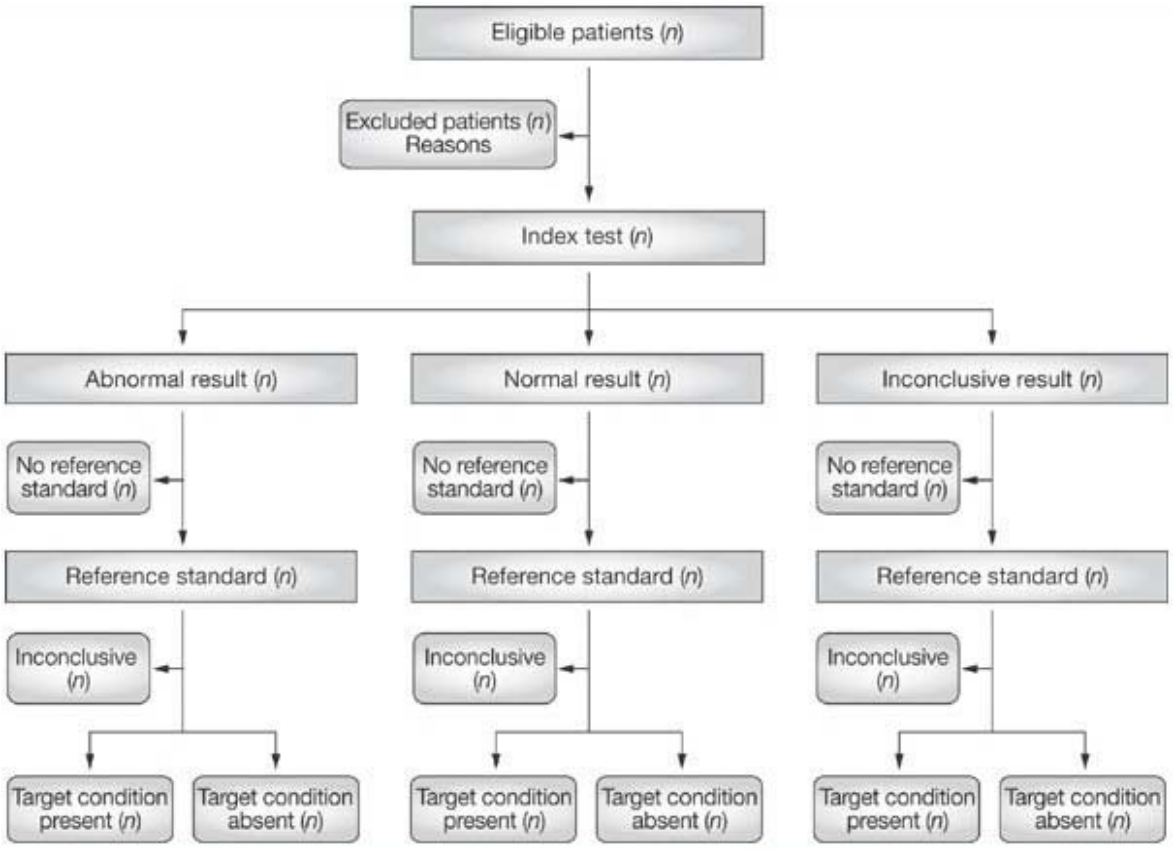


Figure 3.9 Flow chart for STARD (Standards for reporting diagnostic accuracy) checklist.

Table 3-3 The QUADAS (Quality assessment of the diagnostic accuracy studies) tool

Item		Yes	No	Unclear
1.	Was the spectrum of patients representative of the patients who will receive the test in practice?	( )	( )	( )
2.	Were selection criteria clearly described?	( )	( )	( )
3.	Is the reference standard likely to correctly classify the target condition?	( )	( )	( )
4.	Is the time period between reference standard and index test short enough to be reasonably sure that the target condition did not change between the two tests?	( )	( )	( )
5.	Did the whole sample or a random selection of the sample, receive verification using a reference standard of diagnosis?	( )	( )	( )
6.	Did patients receive the same reference standard regardless of the index test result?	( )	( )	( )
7.	Was the reference standard independent of the index test (i.e. the index test did not form part of the reference standard)?	( )	( )	( )
8.	Was the execution of the index test described in sufficient detail to permit replication of the test?	( )	( )	( )
9.	Was the execution of the reference standard described in sufficient detail to permit its replication?	( )	( )	( )
10.	Were the index test results interpreted without knowledge of the results of the reference standard?	( )	( )	( )
11.	Were the reference standard results interpreted without knowledge of the results of the index test?	( )	( )	( )
12.	Were the same clinical data available when test results were interpreted as would be available when the test is used in practice?	( )	( )	( )
13.	Were uninterpretable/ intermediate test results reported?	( )	( )	( )
14.	Were withdrawals from the study explained?	( )	( )	( )

The principles applied by us in designing the diagnostic study included:

1. Study design
   1.1. The study should be of prospective cohort design, recruiting all the patients with the suspected condition;
   1.2. The included patients must have a similar clinical presentation that requires further discrimination by the investigated (index) test and are representative of the patients who will receive the test in practice, to minimise selection and spectrum bias;
   1.3. The participants should be recruited from the consecutive list of candidates to minimise selection bias;
2. Reference standard
   2.1. The selected reference test (reference standard) should be a standard test implemented routinely in clinical practice known to accurately diagnose the condition, to minimise diagnostic bias;
   2.2. All the participants must undergo testing by the reference standard, to minimise and partial verification bias;
   2.3. The time between an index and a reference test should be minimal (preferably up to several weeks' time) to minimise disease progression bias;

2.4. The same reference standard must be used across the study, to minimise differential verification bias;

2.5. The reference standard should be independent of the index test to minimise incorporation bias;

2.6. The execution and interpretation of the reference standard should be performed without knowledge of the results of the index test and vice versa, to minimise diagnostic review bias;

2.7. If index test is performed by several operators, the results of inter-observer variation should be reported to assess inter-observer bias;

2.8. The quality parameters of the laboratory or inter-and intra-experimental variability should be performed;

3. Approach to the data

3.1. The same clinical data should be available when the index test results are interpreted as would be available when the test is used in practice, to minimise clinical review bias;

3.2. Both intermediate results (when index test is not clearly positive or negative) and non-interpretable results (if a test is unable to be performed or measured) should be reported, to minimise diagnostic performance bias;

3.3. Withdrawals from the study should be reported and explained, to minimise diagnostic performance bias.

Chapter 4
Expression profile of plasma miRNAs across the menstrual cycle in women with and without endometriosis

4.1 Introduction

Given the deficiencies associated with current techniques of diagnosis, the ability to utilise a novel biological parameter to assist in the identification of endometriosis would be highly advantageous. As an initial step towards development of miRNA-based test, we aimed at identification of endometriosis-specific miRNA profile in plasma and assessment of the menstrual cycle phase effect on the expression pattern of plasma miRNAs in reproductive-aged women. Supra-physiological hormone levels generated in *in vitro* experiments result in aberrant miRNA expression (180, 197, 209, 233, 236-237), however the physiologic effect of reproductive steroids on miRNA signatures in humans is only starting to be delineated. In eutopic endometrial tissues, the cycle phase impacts significantly on the miRNAome; conversely this tissue undergoes dynamic, hormone- dependent, morphological changes that are likely to be regulated by miRNAs (235, 240-241). We measured circulating miRNA levels at 3 different phases of the menstrual cycle in healthy asymptomatic volunteers and in women with endometriosis, to determine whether hormonal fluctuations across the menstrual cycle could confound miRNA profiling. We expected this information would also reveal the best cycle phase to reliably measure blood miRNA biomarkers for endometriosis and other diseases. Finally a functional bioinformatics analysis of circulating endometriosis-associated miRNAs was performed, to delineate their potential biological effects in women with this disabling disease.

4.2 Materials and Methods

4.2.1 Study participants

In this prospective case control study, women with pelvic pain were recruited from the outpatient gynaecology clinics prior to undertaking a diagnostic laparoscopy and only women with endometriosis confirmed at surgery were included. The women in the control group were asymptomatic healthy volunteers who responded to an advertising poster, who did not suffer from pelvic pain and had no family history of endometriosis. All women were between 18-45 years of age, had regular natural menstrual cycles and no record of chronic disease. Women were excluded if they had symptoms of acute infection or allergic reaction within 2 weeks of blood collection. The severity of endometriosis was classified according to the rASRM classification system. Every surgical team included one of three senior gynaecologic

laparoscopists with more than 10 years' experience in the diagnosis and management of endometriosis to minimise diagnostic inaccuracy.

4.2.2 Blood collection and miRNA extraction

Blood was collected at 3 phases of the menstrual cycle: early proliferative phase (day 3-5 of the menstrual cycle with circulating E2 < 265 pmol/L and P4 < 4 nmol/L), late proliferative phase (day 9-12, E2 = 350-1500 pmol/L and P4 < 4 nmol/L) and mid secretory phase (day 18-24, E2 > 150 pmol/L and P4 > 12 nmol/L). For women with pelvic pain, blood tests were taken before and within 2 months of surgery, which was performed between February 2010 and May 2011. The detailed description of blood processing, miRNA extraction and quality assessment of the samples are presented in Chapter 3.

4.2.3 MiRNA profiling

TLDA A and B v.2.0 were used for a screening of miRNA expression and TaqMan miRNA assays were used for validation of the array findings. All samples were processed under similar conditions and run in single replicate for the array experiment and in triplicate measurements for the verification qRT-PCR experiments, as presented in Chapter 3.

4.2.4 Identification of predicted miRNA targets and *in silico* functional analysis

The detailed description of identification of predicted targets and *in silico* functional analyses is presented in Chapter 3.

4.3 Results

4.3.1 Study participants

A total of 16 women, 8 women with endometriosis and 8 healthy controls met the inclusion criteria and were included in the study. Patient characteristics are summarised in Table 4-1. Menstrual cycle characteristics were comparable. The average age of women with endometriosis was significantly lower than the control group (33.4 ± 5.6 vs. 39.5 ± 4.8, p = 0.035), however this difference was of no clinical significance. The patients in both groups designated themselves as Caucasians. Four of the women in endometriosis group suffered from chronic pelvic pain and 4 women suffered from pelvic pain and infertility. Five women were diagnosed with minimal-mild endometriosis (rASRM stage I-II), and 3 women were diagnosed with moderate-severe endometriosis (rASRM stage III-IV). All women had pelvic

endometriosis and 2 women had both pelvic and ovarian involvement. The extent of surgery varied depending on the disease stage from minimal cauterisation to extensive excision and all blood test were taken before a surgical treatment commenced. Three women in the control group underwent surgical sterilisation 6 months preceding recruitment and had a surgical confirmation of normal pelvis. No significant differences in endocrine profiles were found between women with and without endometriosis (data not shown). None of the patients that underwent surgery had other pelvic pathologies, such as fibroid uterus, hydrosalpinx, ovarian masses (other than endometriomas), inflamed appendix or pelvic inflammatory disease.

Table 4-1 Characteristics of the study population

Characteristic	Endometriosis n = 8 patients (23 blood samples)	Healthy Controls n = 8 patients (24 blood samples)	p-value
Age (years), mean ± SD (range)	33.4 ± 5.6 (25-43)	39.5 ± 4.8 (30-44)	0.035
Surgical inspection of pelvis/abdomen, n (%)	8 (100%)	3 (37.5%)	
Endometriosis stage (rASRM), n (%)			
Minimal-Mild (I-II)	5 (62.5%)		
Moderate-Severe (III-IV)	3 (37.5%)		
Indications for surgery, n (%)			
pain	4 (50%)		
pain + infertility	4 (50%)		
sterilisation		3 (100%)	

4.3.2 Performance of the miRNA TLDA

The miRNA across the plasma samples accounted for 41%-84% of total small RNAs with a concentration range of 255-530 pg/µl.

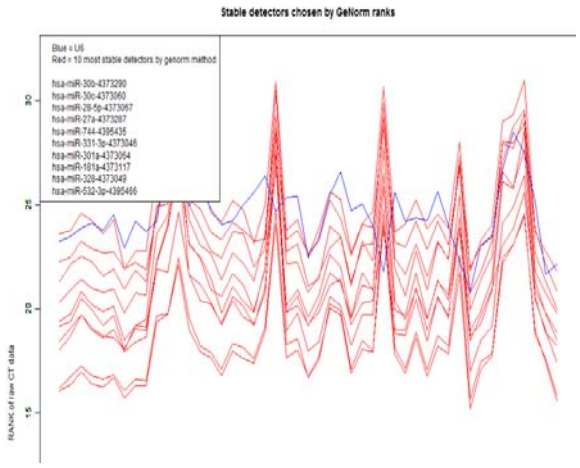
In total, 674 miRNAs were screened (381 on card A in 47 plasma samples and 293 on card B in 37 plasma samples). Ath-mir159a, a miRNA unrelated to mammalian species and used as a negative control on both array cards, was present in 2 samples on card A and in 1 samples on card B, at borderline Cq values of 31.6, 29.9, 31.5, respectively. Mammalian U6 was used as a positive control, all the samples were positive for the replicates of U6 on both cards.

Of the 502 miRNAs detected in at least one sample (316 miRNAs on card A and 186 miRNAs on card B), 128 displayed consistent expression across all samples (104 on card A and 24 on card B). A total of 370 miRNAs were detected in above 50% of the samples (241 miRNAs on card A and 129 miRNAs on card B).

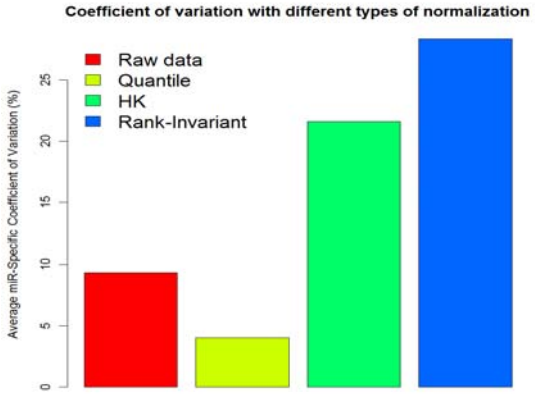
Out of the 47 samples of array card A, 8 samples exhibited less than 50% detected targets. The remainder samples contained 50%-70% of the detected targets. Out of the 37 samples of array card B, 1 sample contained less than 10% of the detected targets, 5 samples 10-20% of the detected targets and the rest of the samples contained 20-48% of the detected targets.

We assumed that “lower abundance” samples might be associated with poorer performance related to technical issues in RNA extraction or RT-PCR experiments, rather than with biological variability. This assumption was supported by the fact that the targets identified on the “low abundance samples” had poorer correlation with the targets on the “high abundance samples”, compared to the correlation between the samples with similar abundance of the expressed targets. The Spearman rank correlation coefficients were above 0.96 for each of the “high abundant” array plates on card A and above 0.86 on card B, above 0.77 for “high abundant” vs. “low abundant” plates on card A and above 0.43 on card B, above 0.83 and 0.78 between the “low abundant” plates on card A and B, respectively; $p < 0.01$. The only plate from card B that contained less than 10% of the expressed targets poorly correlated with other samples ($r_s = 0.15\text{-}0.54$, $p < 0.01$) and therefore was excluded in the analysis.

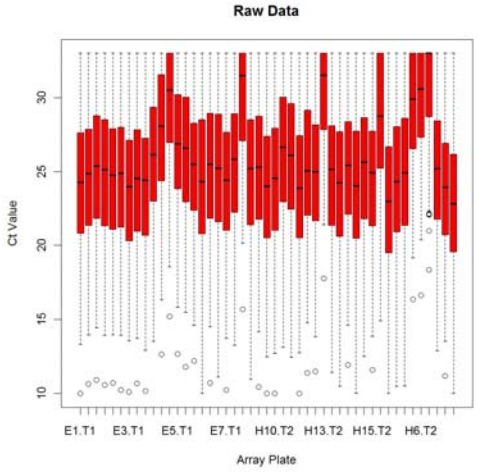
Putative endogenous controls were identified by the GeNorm algorithm. A pair-wise comparison determined that up to 2 endogenous controls were sufficient to normalize expression levels for each of the array cards (V 2/3 = 0.012 for card A and V 2/3 = 0.008 for card B). Interestingly, mammalian U6, suggested by most as a stable and reliable endogenous control, had the highest M-value and was less abundant compared to the other miRNAs identified by GeNorm, therefore it did not appear to be a suitable endogenous control for our data. The selection of the optimal normalisation method was based on comparison of variability measures in the data normalised in different ways, including coefficient of variance, spread of the data and a visual pair-wise comparison between the different normalised profiles. Quantile normalised data expressed less variability and was selected as a method of choice for normalisation in the array experiment (Figure 4.1).



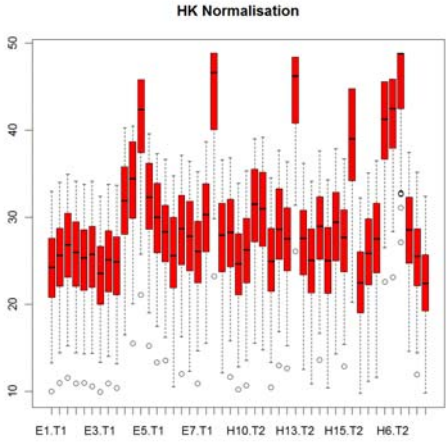
(A)



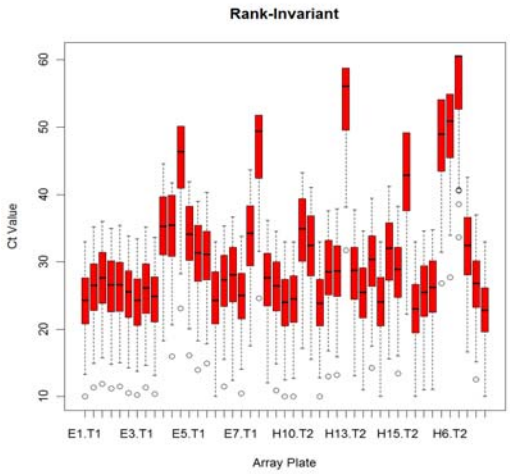
(B)



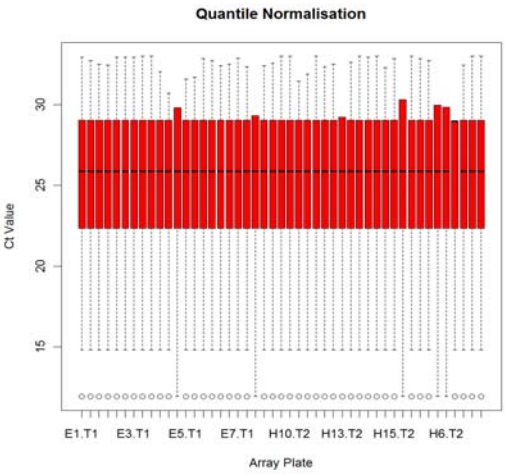
(C)



(D)



(E)



(F)

Figure 4.1 Variability measures of the data, comparison between normalisation approaches: not normalised data (raw data), quantile normalisation, rank-invariant normalisation and normalisation to the endogenous controls identified with GeNorm algorithm (HK). Data from the array cards A. Panel A represents raw Cq values of the stable detectors chosen by GeNorm ranks (red), comparison with Mammalian U6 (Blue). Panel B represents coefficients of variation (CV); Panels C-F represent a degree of dispersion and skewness in the data demonstrated by the box plots, via presenting a sample minimal and maximal observations, median as well as lower and upper quartile; each box-plot corresponds to one array.

4.3.3 Expression of the miRNAs across the menstrual cycle

First, we compared the relative expression of miRNAs between different menstrual cycle stages separately in the endometriosis and in control groups. The list of the top ranked miRNAs identified by LIMMA within each group is presented in Table 4-2. None of the miRNAs exhibited differential expression between the stages of the cycle at a significance level of $p < 0.01$, either in women with endometriosis, or healthy controls. There were certain stages of the cycle where some of these miRNAs had a p-value of $0.01 < p < 0.05$ without a correction for multiple testing. For example, miR-138 had a p-value of 0.038 in healthy controls when late proliferative phase was compared with mid luteal, see Table 4-2. Similar results were obtained when a broader range of the targets were included in the analysis, i.e. targets expressed in at least one sample or when the detection limit cut-off was set at Cq > 35 (data not shown).

Table 4-2 List of the top ranked miRNAs identified across the menstrual cycle in disease-free controls and in women with endometriosis*

ID	A mean[1]	t[2] T1-T2	t[2] T1-T3	t[2] T2-T3	P[3] T1-T2	P[3] T1-T3	P[3] T2-T3	F[4]	F. p-value[5]
Healthy controls									
miR-212	28.00	-1.15	1.48	2.64	0.255	0.145	0.012	3.50	0.039
miR-138	29.06	2.34	0.20	-2.14	0.024	0.843	0.038	3.35	0.044
miR-150	18.06	0.52	2.32	1.80	0.604	0.025	0.079	2.97	0.062
let-7g	21.23	1.56	-0.76	-2.32	0.126	0.452	0.025	2.79	0.072
miR-642	29.68	1.69	2.23	0.54	0.098	0.031	0.590	2.72	0.077
miR-135a	29.79	1.61	2.08	0.47	0.114	0.043	0.641	2.38	0.104
Endometriosis									
miR-141	29.48	-2.54	-0.91	1.66	0.015	0.367	0.104	3.29	0.046
miR-205	30.66	0.53	2.49	1.88	0.598	0.017	0.067	3.40	0.042
miR-130b	24.74	-0.67	-2.22	-1.48	0.505	0.031	0.147	2.58	0.087
miR-331-3p	20.39	-1.12	-2.08	-0.89	0.269	0.043	0.377	2.17	0.126
miR-382	23.54	-0.46	-2.06	-1.53	0.646	0.045	0.133	2.31	0.111
miR-511	29.67	1.09	-0.97	-2.03	0.283	0.336	0.049	2.05	0.141
miR-939	31.69	-2.40	-1.55	0.81	0.022	0.129	0.425	3.00	0.062

* Results of the analysis by using linear models with application of moderated t statistics and nested F-test approach (LIMMA package); separate analysis for women with endometriosis and healthy controls across the three time points of the menstrual cycle;

[1] **A mean** – average normalised Cq values for each probe over all the arrays;

[2] **t** – empirical Bayes T statistics;

[3] **p-value** – p-value corresponding to T statistics;

[4] **F** – F statistics;

[5] **F p-value** – p-value corresponding to F statistics; Statistical significance is determined by $p < 0.01$

C – controls, **E** - endometriosis, **T1** – early proliferative stage of the cycle, **T2** – late proliferative stage of the cycle, **T3** – mid-luteal stage of the cycle.

4.3.4 Differential expression of circulating miRNAs in endometriosis

Control and endometriosis plasma samples were compared at each independent cycle stage. Three differentially expressed miRNAs (miR-23a, miR-574-3p, miR-662) passed the correction for multiple testing at, at least one stage of the cycle. An additional 9 miRNAs were significantly differentially expressed ($p < 0.01$) but did not pass the correction for multiple testing. Overall, our analysis revealed 12 miRNAs that differed between the endometriosis and control groups ($FC > 1.5$, $p < 0.01$), of which 7 miRNAs exhibited differences in expression in the early proliferative stage of the cycle, 10 miRNAs in the late proliferative stage of the, and 6 miRNAs in the mid luteal stage of the cycle (Table 4-3). Five of the miRNAs remained differentially expressed at all 3 stages of the menstrual cycle (Figure 4.2A). In total, 6 miRNAs were down-regulated (miR-23a, miR-574-3p, miR-224, miR-425, miR-29a*, miR-376a*) and 6 miRNAs were up-regulated in endometriosis (miR-155, miR-128, miR-362-3p, miR- 598, miR-93*, miR-662).

Surprisingly, RNU-6B also known as U6 was identified as being significantly differentially expressed in the plasma of women with endometriosis. This supports others observations that RNU-6B does not exhibit stable expression across the samples and should not be used as a reliable endogenous control (361). This small nuclear non coding 45 nucleotide long RNA [Hugo Gene Nomenclature Committee ID HGNC:10228, official symbol RNU6-2, GeneBank accession:K03099, RefSeq accession: NR_002752; sequence (CTGCGCAAGGATGACACGCAAATTCGTGAAGCGTTCCATATTTTT)] does not belong to any known miRNA family and was excluded from the functional analyses and validation experiments.

Another 45 miRNAs showed a trend for significance ($0.01 < p < 0.05$) in differential expression between at least at one of the stages of the menstrual cycle and were considered as a group with borderline pattern of dysregulation (19 in early proliferative stage, 19 in late proliferative stage and 19 in mid luteal stage) (Figure 4.2B). These miRNAs all showed a similar expression pattern across the stages, which was not altered by the stage of endometriotic disease.

Table 4-3 Differentially expressed miRNAs, wide-scale profiling with TLDA, comparisons by the phase of menstrual cycle*

ID	Endometriosis				Healthy controls							
	Mean[1]	SEM[2]	Median[3]	95% CI[4]	Mean[1]	SEM[2]	Median[3]	95% CI4	p-value[5]	adj.p-value[6]	FC[7]	B[8]
T1 Early proliferative stage of menstrual cycle												
miR-23a	29.07	0.37	28.68	0.02	26.81	0.31	26.71	0.02	< 0.001	0.017	-4.76	1.49
miR-574-3p	25.23	1.39	27.44	0.09	20.76	0.38	20.93	0.02	0.003	0.241	-22.16	-1.62
miR-155	23.70	0.31	23.61	0.02	28.87	1.61	30.92	0.10	0.003	0.241	3.60	-1.70
miR-93*	22.15	0.17	22.19	0.01	23.81	0.31	23.92	0.02	< 0.001	0.057	3.16	-0.02
miR-662	28.66	0.60	28.62	0.04	31.38	0.70	31.95	0.04	0.001	0.062	6.59	-0.71
miR-29a*	32.23	0.18	32.17	0.01	30.48	0.38	30.48	0.02	0.003	0.109	-3.36	-1.57
miR-376a*	32.32	0.22	32.39	0.01	30.87	0.57	30.76	0.04	0.010	0.251	-2.71	-2.74
T2 Late proliferative stage of menstrual cycle												
miR-23a	29.38	0.32	29.17	0.02	27.26	0.38	26.94	0.02	< 0.001	0.047	-4.32	0.33
miR-574-3p	26.27	1.44	26.90	0.09	20.67	0.35	20.44	0.02	< 0.001	0.047	-48.50	-0.04
miR-128	24.42	0.27	24.16	0.02	26.03	0.47	25.62	0.03	0.001	0.101	3.05	-1.10
miR-155	23.44	0.31	24.00	0.02	28.94	1.71	31.32	0.11	0.002	0.142	45.25	-1.67
miR-224	25.53	0.29	25.68	0.02	24.45	0.19	24.22	0.01	0.006	0.239	-2.11	-2.48
miR-362-3p	28.31	0.11	28.29	0.01	29.42	0.37	29.25	0.02	0.006	0.239	2.16	-2.51
miR-598	25.04	0.11	24.94	0.01	26.03	0.48	25.56	0.03	0.007	0.240	1.99	-2.65
miR-662	28.53	0.11	28.65	0.01	31.89	0.66	32.97	0.04	< 0.001	0.019	10.34	0.93
miR-93*	22.62	0.11	22.52	0.01	24.07	0.34	24.09	0.02	0.003	0.175	2.73	-1.70
miR-29a*	32.06	0.34	32.35	0.02	30.48	0.34	30.41	0.02	0.009	0.362	-2.99	-2.70
T3 Mid-secretory stage of menstrual cycle												
miR-155	23.36	0.36	23.25	0.02	28.54	1.62	30.21	0.10	0.003	0.408	36.25	-1.85
miR-574-3p	25.04	1.63	27.34	0.10	20.68	0.28	20.97	0.02	0.003	0.408	-20.68	-1.95
miR-23a	29.22	0.36	29.32	0.02	27.74	0.47	27.44	0.03	0.006	0.427	-2.77	-2.40
miR-425	22.47	0.24	22.29	0.02	21.64	0.16	21.69	0.01	0.007	0.427	-1.77	-2.47
miR-662	28.66	0.26	28.83	0.02	31.93	0.51	32.42	0.03	< 0.001	0.041	9.65	0.26
miR-93*	22.58	0.11	22.76	0.01	23.92	0.41	23.68	0.03	0.007	0.453	2.51	-2.49

*Results of the analysis using linear models with application of moderated t statistics (LIMMA package); comparison between women with endometriosis and healthy controls separately at each time point of menstrual cycle;

[1]**Mean** - average normalised Cq values within a group;

[2] **SEM** - standard error of the mean;

[3]**Median** – median normalised Cq within a group;

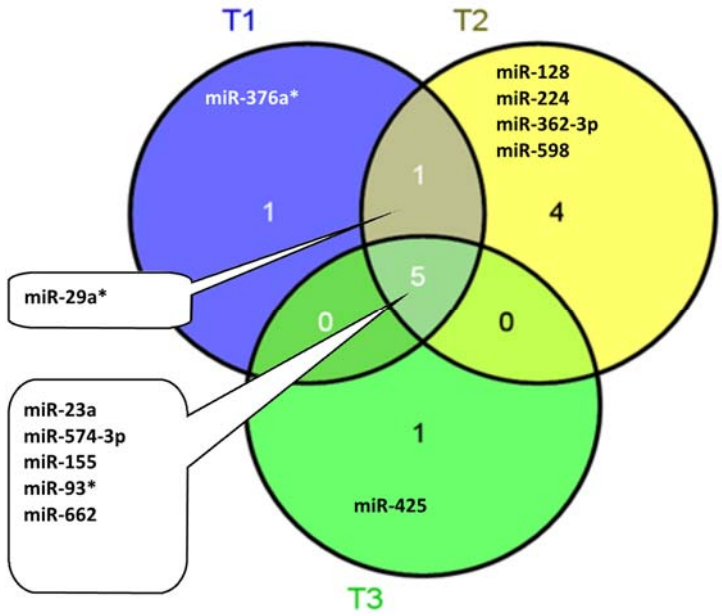
[4]**95% CI** – 95% confidence interval of the median miRNA expression within a group;

[5]**p-value** – p-value corresponding to T statistics;

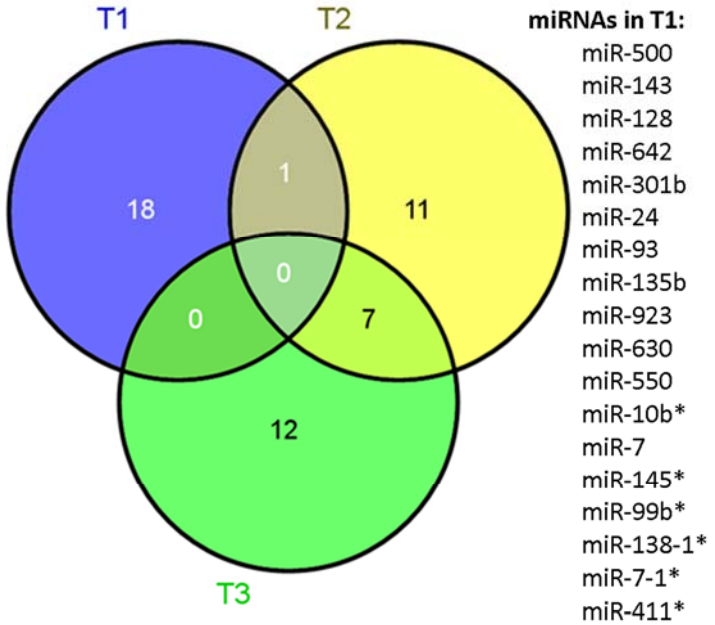
[6]**adj. p-value** – p-value after adjustment for multiple testing by Benjamini and Hochberg's method;

[7]**FC** – fold change. Negative FC designates downregulated miRNAs, positive FC – upregulated miRNAs. FC represents "least FC";

[8]**B** –log-odds that the target is differentially expressed (B statistics).



(A)



miRNAs in T1:
miR-500
miR-143
miR-128
miR-642
miR-301b
miR-24
miR-93
miR-135b
miR-923
miR-630
miR-550
miR-10b*
miR-7
miR-145*
miR-99b*
miR-138-1*
miR-7-1*
miR-411*

miRNAs in T2:
miR-320
miR-369-3p
miR-19b
miR-181c
miR-654-3p
miR-30b
miR-495
miR-378*
miR-661
RNU6B
miR-625*

miRNAs in T3:
miR-200b
miR-362-3p
miR-331-3p
miR-505
let-7g
miR-29c
miR-433
miR-31
miR-130b
miR-487b
miR-379*
miR-526b*

miRNAs in T2 and T3:
miR-148b
miR-532-5p
miR-148a
miR-874
miR-452
miR-502-3p
miR-1

(B)

Figure 4.2 Venn diagram representing a comparison between lists of differentially expressed miRNAs in endometriosis, according to the phase of menstrual cycle (T1, early proliferative phase; T2, late proliferative phase; T3, mid luteal phase). Panel A represents significantly differentially expressed miRNAs ($p < 0.01$), panel B represents comparison of the miRNAs that exhibited trend for significance in differential expression in endometriosis ($0.05 > p > 0.01$).

Hierarchical clustering broadly demonstrated that endometriosis patients cluster separately from non-endometriosis controls as expected (Figure 4.3). However the individual was a stronger influence on clustering than disease status and cycle phase. This supported our initial findings that none of the endometriosis- associated miRNAs were cycle phase dependent.

Notably, a subgroup of 3 control patients clustered separately with 3 endometriosis patients. This was an unexpected finding. It could not be explained by the technical issues as all samples were handled in a similar way by the same investigators and had similar quality of miRNA. The hormonal and demographic profile of separately clustering endometriosis patients was comparable to other endometriosis sufferers. Two of them had mild endometriosis (rASRM I-II) and one had severe disease (rASRM III-IV) and none of them had additional pelvic pathology or a history of any other medical condition that could confound the findings. Similarly, 3 of the non-endometriosis controls that exhibited similarity of molecular profile with the above endometriosis subgroup did not have any features discriminating them from the rest of the control group. The average age in this subgroup was higher than for other controls (38 vs. 33.3); however the age difference was comparable to the one with endometriosis group (33.4±5.6) and complied with the study inclusion criteria.

A subgroup analysis excluding these patients was performed to determine if there was significant confounding by these patients on the dataset. As in the original analysis, none of the miRNAs demonstrated variations in expression across the menstrual cycle. However, the number of the differentially expressed miRNAs at $p < 0.01$ was increased ($n = 37$) and within the subgroup analysis 8 rather than 3 miRNAs now passed the correction for multiple testing (data not shown). As the heterogeneity of our samples is likely to represent true biological variability and the original analysis conservatively captured the most significantly dysregulated miRNAs, we continued to explore the main dataset.

The results of the analysis on the data with a higher detection limit cut off ($Cq > 35$) or when miRNAs present only in 1 sample were included, showed substantial overlap with the reported results, suggesting that the choice of the most significantly differentially expressed miRNAs was not substantially influenced by the ways the data was handled (data not shown).

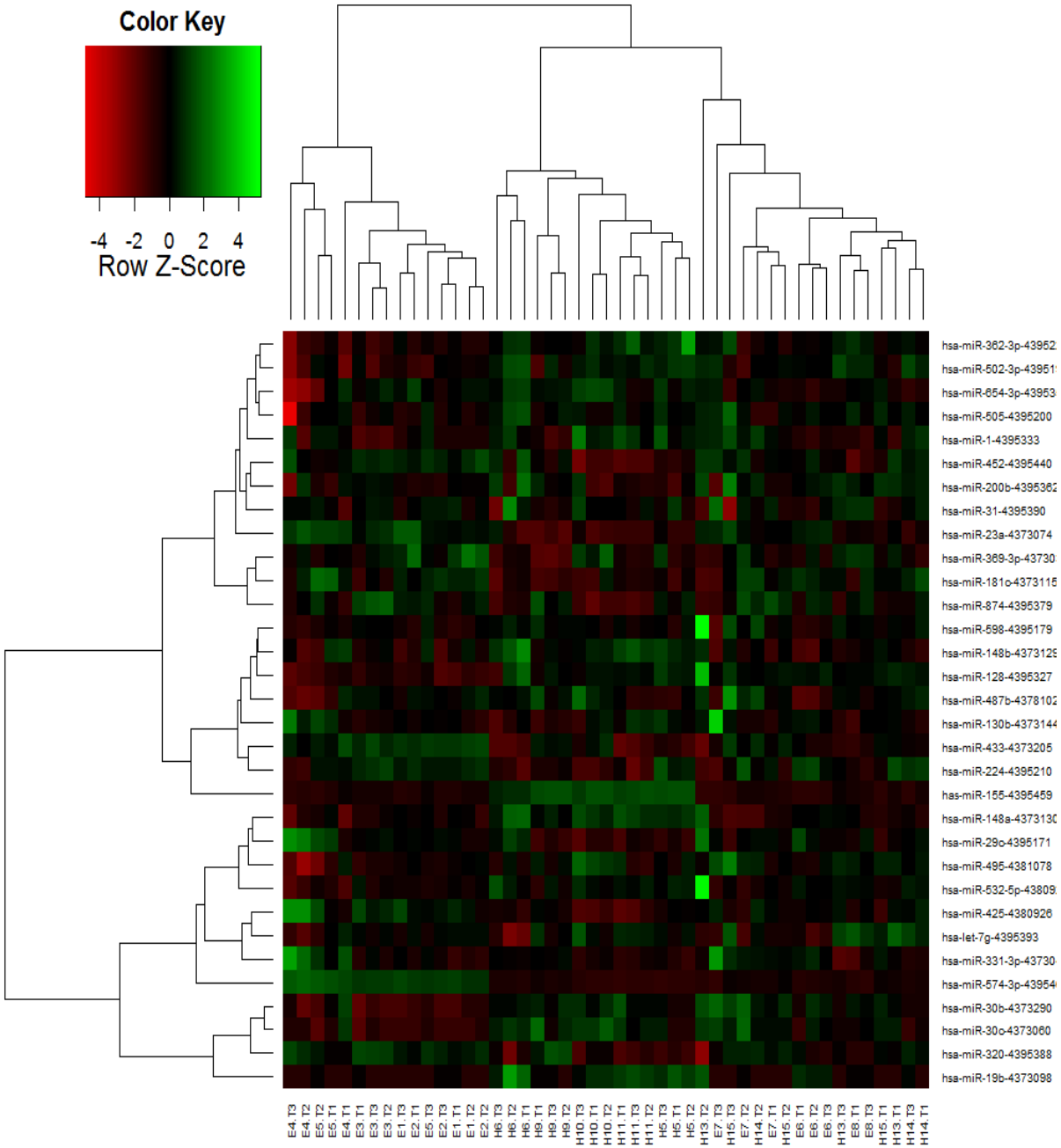
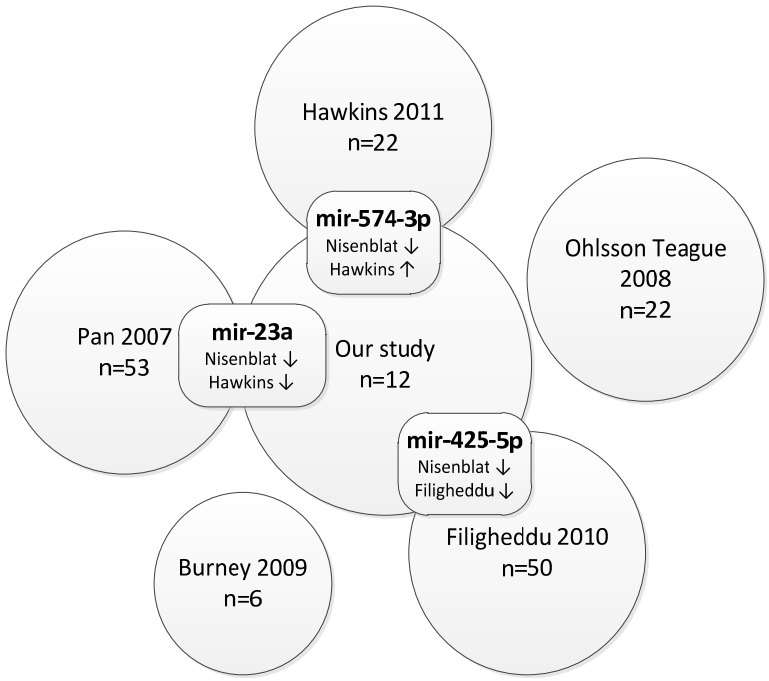


Figure 4.3 Heat map analysis representing the top ranked plasma miRNAs dysregulated in endometriosis. The analysis is based on p-values generated with the moderate t statistics. The rank of the miRNAs is determined by clustering based on the similarity of the miRNA expression patterns.

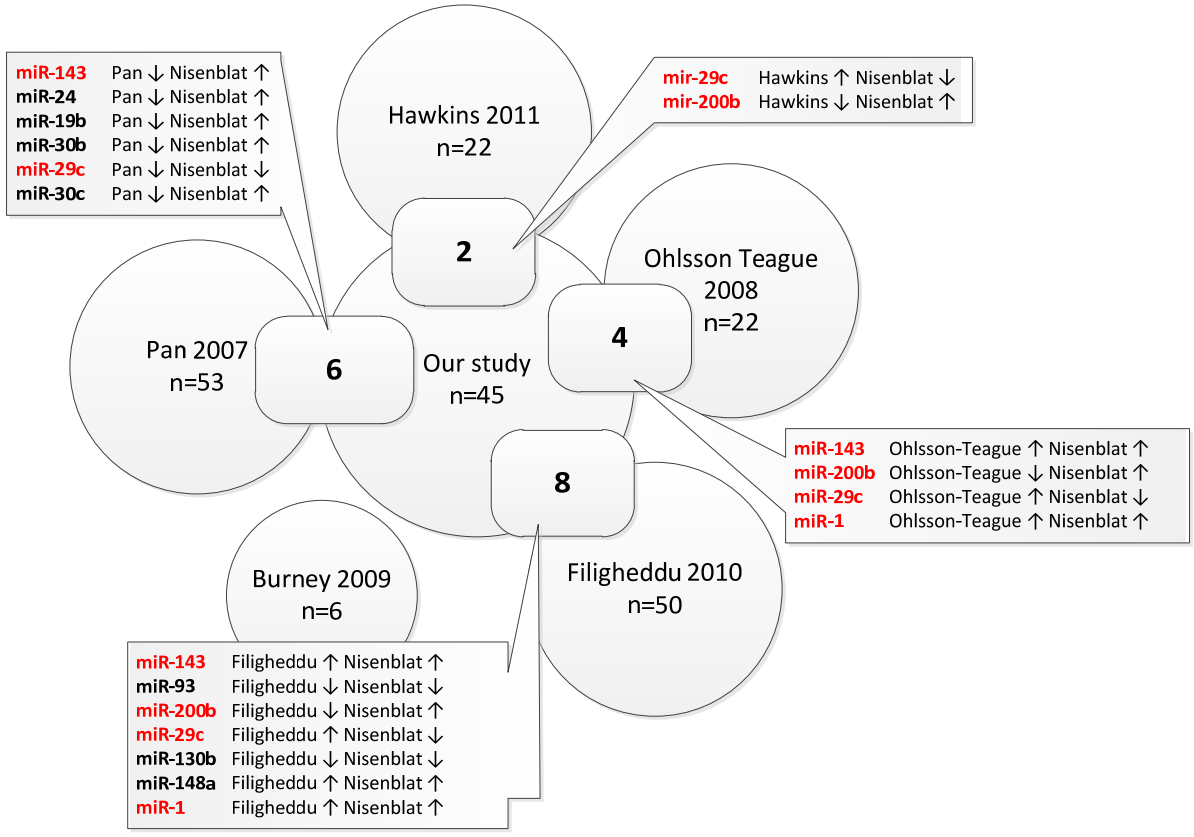
4.3.5 Correlation of plasma miRNA profile in endometriosis with previously published data on miRNA signatures in endometrial tissue

To explore whether plasma miRNAs correlate with the earlier established endometriosis-specific miRNA profile in the endometrium, we compared the list of differentially expressed miRNAs with the previously published data from five large-scale experiments. These studies

explored miRNA profiles in eutopic and/or ectopic endometrial tissue in women with and without endometriosis, by using deep sequencing (205) or hybridisation microarray platforms (206-209).



(A)



(B)

Figure 4.4 Comparison of the differentially expressed plasma miRNAs in endometriosis with previously published data on miRNA profile in endometrial tissue (eutopic and ectopic endometrium). Panel A represents comparison with 12 significantly dysregulated miRNAs, panel B – comparison with 45 miRNAs exhibiting trend for dysregulation.

Three of the 12 differentially expressed plasma miRNA were previously identified as dysregulated in endometriosis in endometrial tissues (Figure 4.4A). The direction of regulation of miR-23a (209) and miR-425-5p (207), but not miR-574-3p (205), was concordant between plasma and endometrial tissue. Of note, 11 out of 45 miRNAs with a trend for significance were previously identified in association with endometriosis and 4 of these miRNAs were identified in more than 1 study (Figure 4.4B).

4.3.6 Validation of the array results with qRT-PCR analysis of miRNA expression

To confirm the comparability of TLDA-based genome-wide profiling with individual quantifications, we further quantified the expression levels of 6 miRNAs using qRT-PCR, namely miR-23a, miR-155, miR-128, miR-662, miR-93* and miR-574-3p that were amongst the top ranked differentially expressed miRNAs. In this experiment we aimed to validate whether experimental variables such as megaplex primer pools and preamplification step as well as different principles of the analyses, e.g. ways of normalisation and statistical methods, had an impact on final conclusions. We carried out the individual TaqMan mature miRNA expression assays on the same group of subsets at 3 time points of the menstrual cycle.

Out of the 6 tested miRNAs that showed differential expression in plasma from endometriosis patients by TLDA analysis, the levels of 4 miRNAs (miR-128, miR-155, miR-662 and miR-93*) differed significantly in this singleplex qPCR at least at one phase of the cycle and when cycle stage was disregarded in the analysis (Figure 4.5). Both miR-662 and miR-93* were dysregulated at each stage of the menstrual cycle. MiR-128 differed significantly at 2 and miR-155 at one stage of the cycle with an observed trend for differential expression at the other cycle stages.

4.3.7 The role of the circulating miRNAs in endometriosis

4.3.7.1 Identification of the predicted miRNA targets and *in silico* functional analysis

To analyse the possible role of the differentially expressed circulating miRNAs in endometriosis, we determined the target mRNAs of the 12 differentially expressed miRNAs by using the IPA Target filter. Experimental confirmation was available for 166 targets of 5 miRNAs and a further 2904 targets for 9 miRNAs were predicted with high level of confidence.

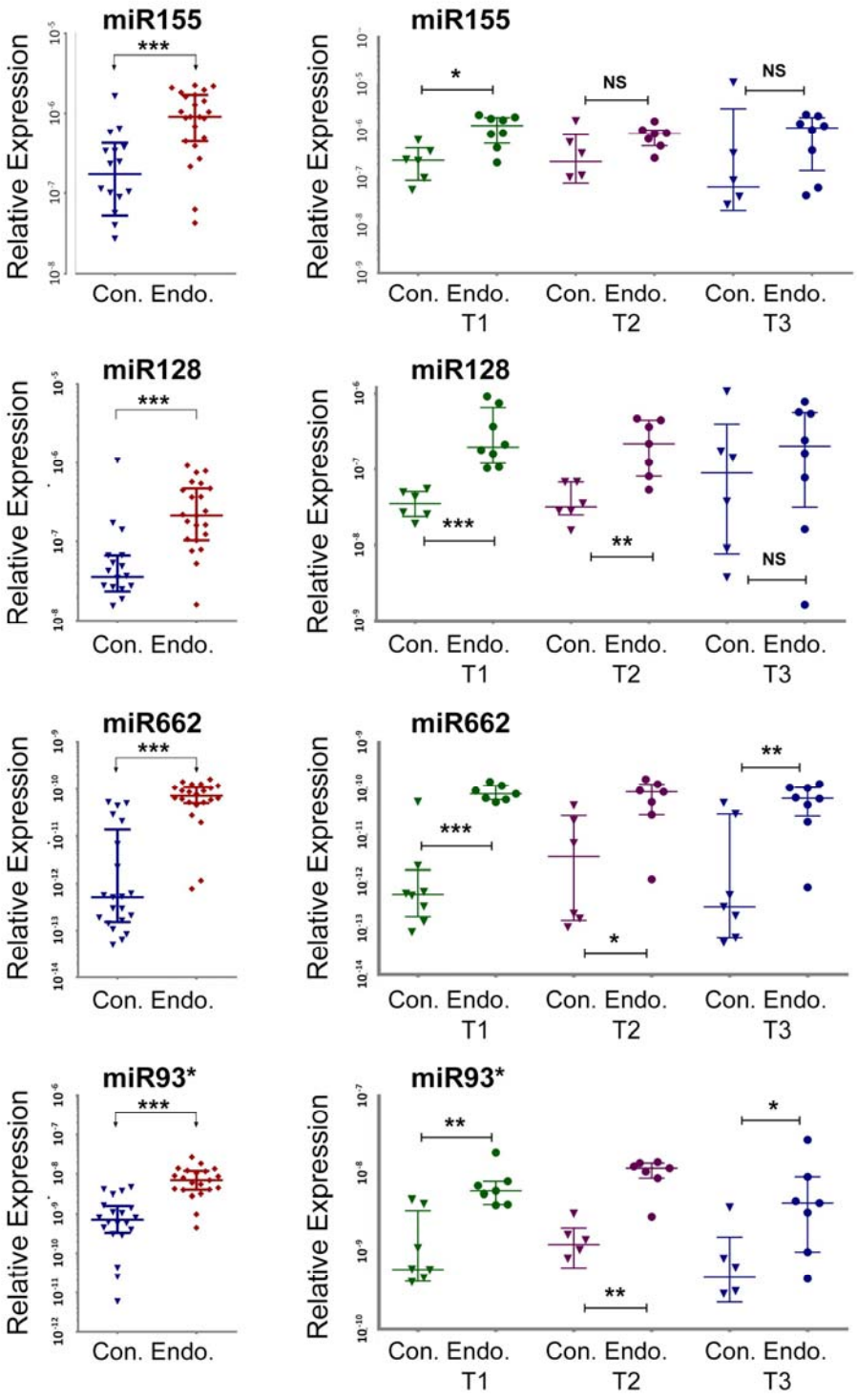


Figure 4.5 Validation of the TLDA data with individual qRT-PCR assays. Expression profiles of the 4 significantly altered miRNAs identified in plasma from women with and without endometriosis (Endo vs. Con, n = 8 in each group) across the 3 phases of menstrual cycle. Left panels: total samples irrespective of cycle phase; right panels: menstrual cycle phase separate comparisons (T1 – early proliferative phase, T2 – late proliferative phase, T3 – mid luteal phase). Data are presented as relative expression values normalised to spiked in cel-miR-54. [Median (middle line), interquartile range (top and bottom lines)], statistical significance (Mann-Whitney U test) was defined as $p < 0.05$. * designates $p \leq 0.001$; **designates $p < 0.01$; *designates $p < 0.05$; NS designates "non-significant".**

To ensure the completeness of the predicted mRNA target lists, we implemented an additional target prediction algorithm (MirTarget2 tool) incorporated in the miRDB database and merged both target datasets. Additional 264 targets predicted with high confidence (score > 80) were identified. Overall, 3144 targets identified for 11 miRNAs with high confidence by both methods (Supplemental Material on the enclosed CD, Table 1S) were subjected to an IPA core analysis. The predicted targets were significantly enriched for several biological functions known to be involved in endometriosis, including: gene expression, cellular movement, cell death, and cellular growth and proliferation. Cancer, haematological disease, reproductive disorders, immunological and inflammatory disease were among the diseases significantly associated with the investigated targets (Figure 4.6A). The top canonical pathways enriched for these predicted targets included molecular mechanisms of cancer, TGF-β signalling, peroxisome proliferator-activated receptor-α/ retinoid X receptor-α (PPARa/RXRα) activation, Wnt/CTNNB1 signalling, and gonadotropin-releasing hormone (GNRH) signalling (Figure 4.6B). Using a score of > 20 as a cut-off, 14 molecular networks were identified by IPA and were constituted by 464 predicted targets. The 2 top networks of gene expression and cell cycle, tissue and embryonic development centre on CTNNB1 [β-catenin [cadherin-associated protein)] and CCND1 (cyclin D1), both of which encode for multifunctional proteins that play essential roles in development and tissue maintenance (Figure 4.6C). Other significant networks of the identified targets converged on NFkB – TGFβR1/2, ER, PR and LH/follicle-stimulating hormone (FSH) all of which are associated with the endometriotic disease process. The full list of the biological functions, canonical pathways and molecular networks associated with the predicted targets are presented as Supplemental Material on the enclosed CD, Table 2S.

4.3.7.2 Agreement between the predicted targets of deregulated circulating miRNAs with validated dysregulated genes in endometriosis

Next, we investigated whether the predicted targets of the differentially expressed miRNAs were in accordance with the results of the previously published mRNA profiles in endometriosis in humans. Four publically available gene micro array datasets have been deposited into the Gene Expression Omnibus http://www.ncbi.nlm.nih.gov/projects/geo; with accession numbers GSE23339 (205) and GSE7305 (84) for endometrioma and eutopic control endometrium, GSE11691 (85) and GSE5108 (83) for ectopic and eutopic endometrium in endometriosis.

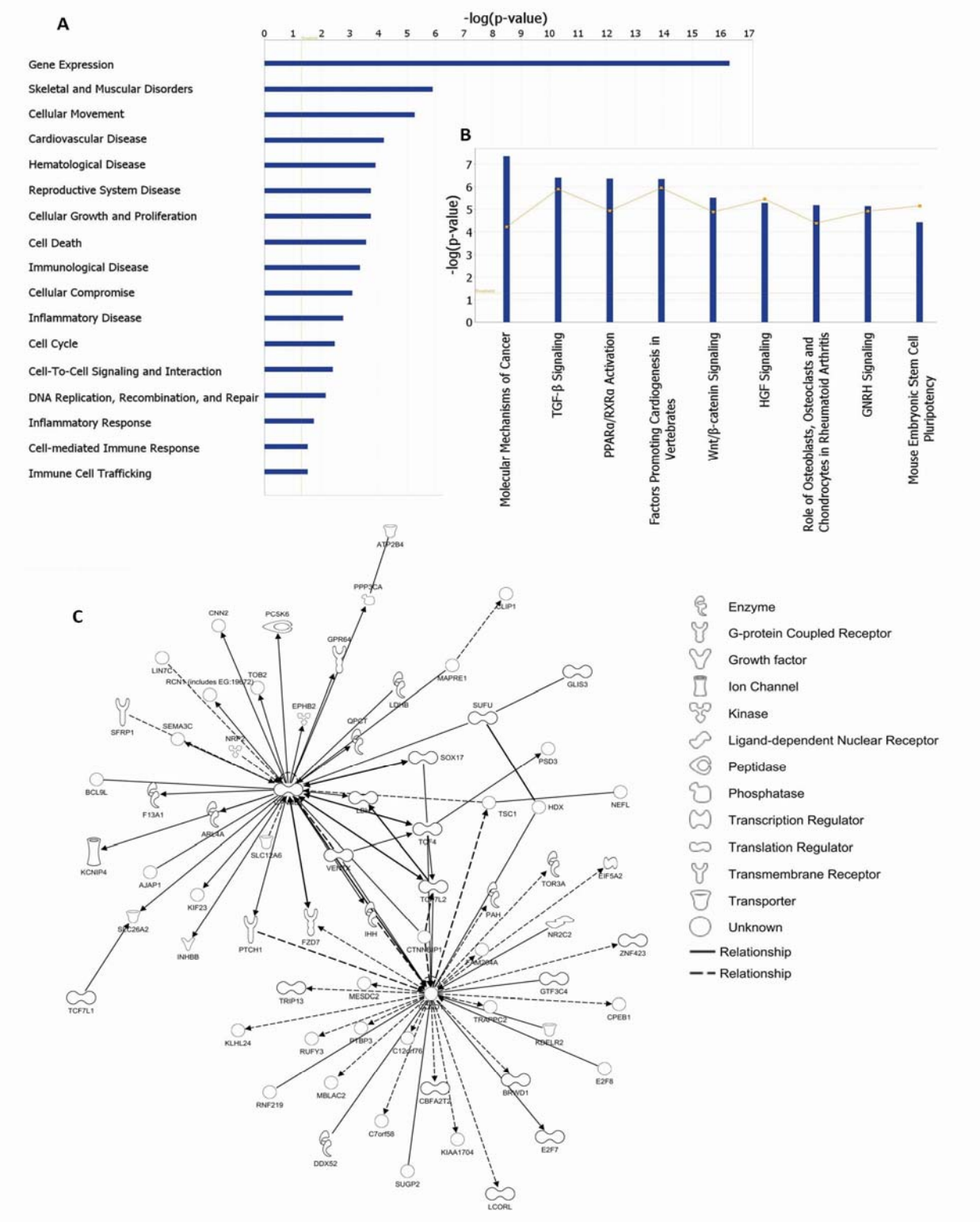


Figure 4.6 Ingenuity Pathways Analysis of the 3144 predicted targets of endometriosis-associated circulating miRNAs. (A) Most significant biological functions; (B) Most significant canonical pathways; (C) Molecular networks enriched by the predicted targets centred around β-catenin (CTNNB1) and cyclin D1 (CCND1): Gene expression, Cell cycle, Tissue development, Renal development and function, Cardiovascular system development and function.

Hypothesising that the differentially expressed genes common to all the above studies are likely to be specific to endometriosis independently of the site of the lesion, we focused only on the list of genes that overlapped between the datasets. Hawkins et al., identified 116 up-regulated and 72 down-regulated genes that intersect the 4 datasets (205). When the intersect of the miRNA predicted target genes from 4 published studies was compared to transcript targets of the differentially regulated plasma miRNAs, 59 overlapping transcripts were identified (39 up-regulated and 20 down-regulated). The list of the mRNAs predicted to be regulated by endometriosis associated plasma miRNAs that were also validated to be dysregulated in endometrial tissue in endometriosis are presented as Supplemental Material on the enclosed CD, Table 3S. The above mRNAs appeared to be predicted targets of the 11 out of 12 miRNAs (with an exception for miR-93-3p). An inverse correlation of expression was observed for some miRNA-mRNA pairs but not for others (Figure 4.7).

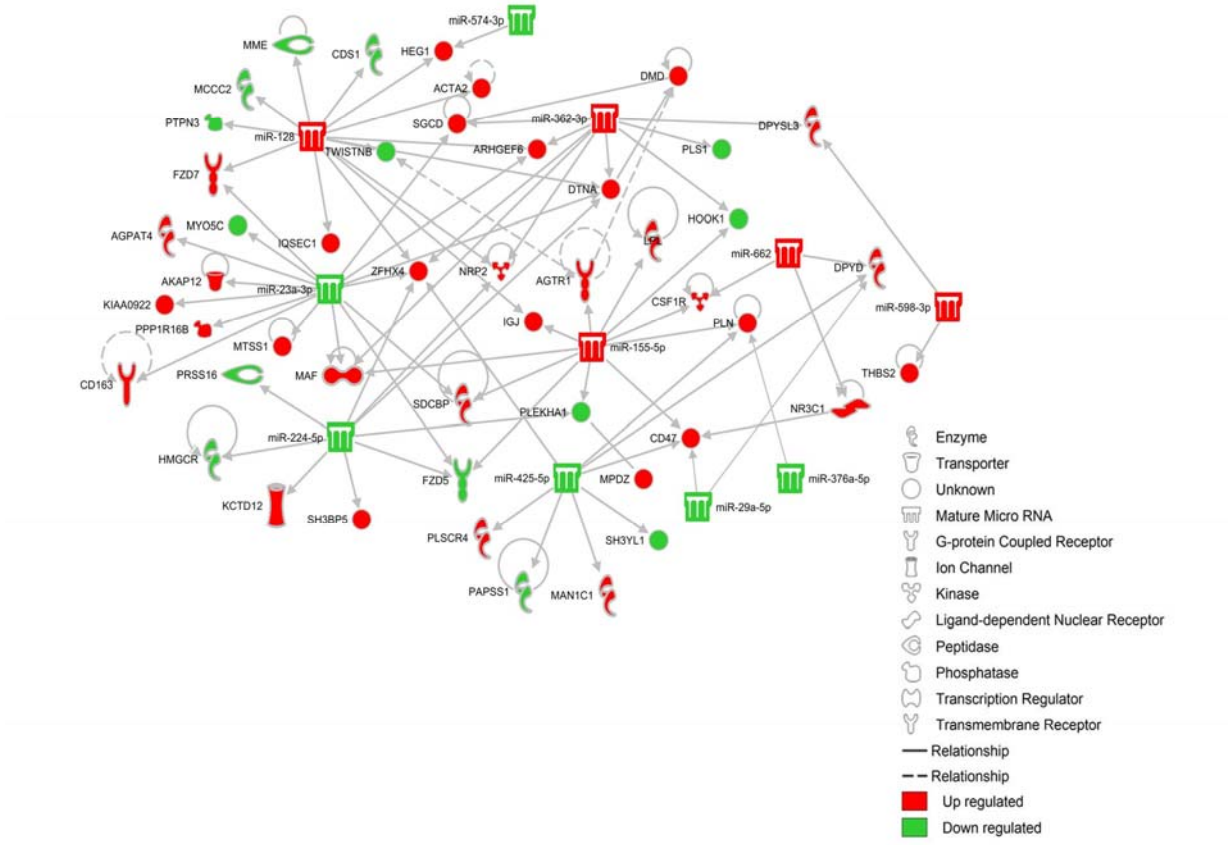


Figure 4.7 Plasma miRNA interactions with endometriosis associated mRNAs (overlapping between the predicted targets identified in the current study and previously experimentally validated transcripts in 4 gene expression studies). Functional activity is listed in figure panel for each transcript; up-regulated (red), down-regulated (green). An expected inverse correlation of expression was observed for some but not all miRNA-mRNA pairs.

An IPA analysis revealed that the top functions enriched in the predicted target genes involved Cellular Function and Maintenance, p = 1.52E-03 - 4.91E-02, Amino Acid Metabolism, p = 3.86E-03 - 3.86E-03, Cell Morphology, p = 3.86E-03 - 3.05E-02, Cell-To-Cell Signalling and Interaction, p = 3.86E-03 - 4.91E-02 and Cellular Assembly and Organization, p = 3.86E-03 - 4.91E-02. The investigated targets were incorporated in 6 molecular networks of cell cycle, cell to cell signalling, cell survival, cellular development and immune cell trafficking. The 3 most significant networks centred on CCND1, NR3C1/TCR, and NFkB/CTNNB1. The list of the biological functions and molecular networks associated with these targets are presented as Supplemental Material on the enclosed CD, Table 4S.

4.4 Discussion

In this study we characterised the circulating miRNA profile in women of reproductive age with and without endometriosis throughout the menstrual cycle. We used an RT-PCR based array platform to assess the miRNA expression in subsets with well characterised hormonal profiles and absence of other pathologies, followed by *in silico* functional analyses of the miRNAs of interest.

4.4.1 Influence of the menstrual cycle on expression of circulating miRNAs

This study revealed differences in plasma miRNA expression between women with and without endometriosis across and at different phases of the menstrual cycle. However, cyclical changes in reproductive steroids appeared to have little impact on plasma miRNA profiles in either healthy control or endometriotic populations. This suggests there is considerable potential to develop a non-invasive diagnostic test using plasma miRNAs, although larger samples numbers are required to optimise diagnostic algorithms, then determine the sensitivity and specificity of the measurements prospectively in a test population.

Global miRNA profiling in whole blood has been performed in healthy reproductive age women and demonstrated age related differences in miRNA plasma levels (362), however no attempt was made to determine if cycle phase confounded the results. To our knowledge, this is the first study to address the impact of the menstrual cycle stage on circulating miRNAs.

The regulation of miRNAs by ovarian steroids and vice versa is well documented in *in-vitro* experiments reviewed in (180, 197, 236-237). Oestrogen-dependent miRNA association with the Drosha complex or inhibition of miRNA maturation at the level of processing pri-miRNAs

are among the suggested mechanisms of this interaction (237-239). E2 and P4, alone or in combination with their antagonists (ICI-182780 and RU486, respectively) altered the expression of miR-20a, miR-21 and miR-26a in isolated endometrial cells (209). MiR-17-5p, miR-23a, miR-23b and miR-542-3p and their targets CYP19A1, StAR, and COX-2 also exhibited reproductive steroid dependence in human endometrial cell lines. As these miRNAs act to promote cell proliferation, it was postulated that they may have a role in promoting endometriotic lesion development, which is enhanced by an estrogenic environment (233).

Kuokkanen et al., identified 24 miRNAs that were differentially expressed when endometrial epithelium from healthy women was analysed in the proliferative vs. secretory phase of the menstrual cycle (235). Predicted targets of the menstrual phase specific miRNAs were identified in a parallel mRNA microarray analysis and were associated with DNA replication, cell cycle progression and cell proliferation. Menstrual phase specific endometrial transcriptome signatures were characterised in several other wide genome mRNA profiling studies (96, 363-364).

In contrast, a miRNA profile sub-analysis of eutopic endometrial tissues failed to demonstrate menstrual phase differences in miRNA expression, although it was confined to a small population of women with endometriosis (208). Other small studies have measured the expression of individual miRNAs in human endometrium across the menstrual cycle and identified cycle specific expression patterns. In one study, miR-135a and miR-135b showed a reduction in endometrial expression levels at the time of ovulation in women with and without endometriosis (240). Ramon et al., reported increased levels of miR-15b, miR-20a and miR-221 in secretory phase eutopic endometrium in healthy controls (241), despite a previous report failing to observe cycle dependence in these miRNAs (235). Interestingly, researchers were able to demonstrate a significant increase in miR-15b, miR-17-5p, miR-20a, miR-125a, miR-221 and miR-222 in secretory compared to proliferative phase endometrioma but did not detect any cycle-related expression differences in eutopic endometrium from women with endometriosis.

The absence of significant menstrual phase variations in plasma miRNA expression does not exclude the existence of miRNA-hormonal interactions nor does it contradict the previous reports of cycle-dependant miRNA pattern in endometrial cells and tissue. Endometrial tissue has a higher local concentration of reproductive steroid hormones than the systemic circulation**,** thus E2 and P4 are likely to have a greater impact on endometrial miRNA profiles.

Secondly, cycle-dependent hormonal changes induce multiple cellular events that are likely to be regulated by and influence the levels of miRNAs (365). The circulatory miRNA profile is comprised of miRNAs that have originated from various tissues and organs in the body. The contribution of endometrial miRNAs may be masked by miRNAs of other origins and their contribution to the cyclicity of miRNAs may not be able to be evaluated. Finally, the power of our study may not have detected small but significant miRNA cycle phase variations in plasma miRNAs and larger studies are warranted to explore this possibility. None of the miRNAs identified in our study as having a trend to cycle phase variation have demonstrated steroid dependence *in vitro*.

Our data indicates that the miRNA profile in reproductive age women is disease-specific, rather than cycle dependent and this applies to any miRNA and not only the ones linked with endometriosis. This information is of particular importance in biomarker research, indicating that the stage of the menstrual cycle in which blood is drawn, or the use of the hormonal medications do not have a negative impact on the performance of a miRNA-based test.

4.4.2 The plasma miRNA profile in endometriosis

This is one of the first studies to identify endometriosis-associated circulating miRNAs, in which, we demonstrated a distinct miRNA expression pattern in the plasma from endometriosis sufferers. The main objective of this study was to substantiate the hypothesis via looking for promising lead markers that need to be followed up further. For this reason we decided to minimise type 2 errors produced by correction for multiple testing, in order to avoid exclusion of potentially important observations even if they may later prove to be false. Therefore, the correction for multiple testing was applied to the data but was not considered to be an eliminating factor in the selection of potentially relevant plasma miRNAs. Three levels of confidence were assigned for observed dysregulated miRNAs: 1 – 'highly significant', which was in keeping with an FDR of up to 5%; 2 – 'significant', for the type 1 error was below 1% without correction for multiple testing, and 3 – 'exhibiting trend for significance', i.e. type 1 error 1-5%, without correction for multiple testing. In this report, miRNAs detected at the first 2 levels were considered significant.

Six plasma miRNAs were up-regulated and 6 down-regulated in endometriosis compared to healthy controls whereas 45 plasma miRNAs showed a trend to significantly differential regulation in endometriosis. Interestingly, although we did not find menstrual cycle dependent miRNA expression levels in either the control or endometriosis groups, 7 of the 12

miRNA transcripts were able to distinguish women with and without endometriosis only at certain phases of the cycle. The late proliferative stage appeared to be the best sampling phase with 10 of the miRNAs demonstrating an ability to distinguish women with endometriosis from healthy controls. It may be that women with endometriosis have a mild divergence from the usual physiological responsiveness to steroid hormones in the late follicular phase that is apparent only when women with and without endometriosis are compared. This could explain why women with endometriosis tend to have a prolonged proliferative phase and are resistant to P4 compared to healthy controls (366).

Surprisingly 3 out of 8 disease-free women clustered with the endometriosis cohort. Notably, all were asymptomatic and had surgical verification of a normal pelvis. It is possible that confounders such as smoking, nutritional status, subclinical infection or toxin exposure influenced the data, despite an extensive history being taken (244, 252). A past history of tubal sterilisation was the only common feature of this group; however blood sampling was performed at least 6 month after surgery. It is possible that a chronic inflammatory host response to necrotic fallopian tube tissue crushed by a Filshie clip could create a shift in miRNA profile even 6 months after application. The inclusion of these patients did not generate false positive findings from the dataset, as exclusion of these patients did not alter the list of differentially expressed miRNAs and improved rather than reduced the significance level of discrimination.

4.4.3 Functional involvement of dysregulated plasma miRNAs in endometriosis

Molecular networks constituted by the endometriosis-associated predicted targets converged on molecules involved in inflammation (NFkB), proliferation (MAPK), tissue remodelling (TGF-β) and steroidogenesis (ER, PR, LH, FSH), which is consistent with our current understanding of the cellular events that occur in endometriosis. The top functional networks converged on CTNNB1, and its downstream target CCND1. CTNNB1 is a key factor in the Wnt signalling pathway and the MAPK and IKKB/NFkB cascades, all of which have a known association with endometriosis (367-368). Enhanced CTNNB1 signalling has been identified in ovarian cancer and is thought to have a role in the malignant transformation of endometriosis (367, 369-370). The MAPK/pERK12 cascade, which has been shown to be up-regulated in endometriosis, was shown to increase CCND1 activity in early secretory endometrial stromal cells from women with endometriosis, which consequently led to an aberrant endometrial proliferation (371).

Several plasma miRNAs identified in the current study are known to regulate CCND1, either directly (miR-155-5p, miR-23a, miR-224-5p, and miR-425-5p), or indirectly by targeting transcription factors that regulate CCND1. For example CCND1 is attenuated by TR4 [NR2C2] (nuclear receptor subfamily 2 group C member 2) and by VENTX (VENT homeobox), which are both repressed by the endometriosis-associated plasma miRNA miR-362-3p. CCND1 is also up-regulated by TCF4 (transcription factor 4) and E2F8 (E2F transcription factor 8), which are repressed by miR-128, miR-155-5p, miR-23a, and by miR-23a respectively. Interestingly, in liposarcoma enhanced CTNNB1/CCND1 activity is also evident and this is linked to over-expression of miR-155 (372)

Many studies have demonstrated that miR-155 can act as an oncogenic miRNA (oncomir), promoting tumourogenesis and cancer progression via translational inhibition of tumour suppressor genes (372-378) such as forkhead box O3 (FOXO3) gene, MNNG HOS transforming gene (MET-proto-oncogene), cysteine kinase 1-α (CK1α), a p53-induced nuclear tumour protein 1, and CCAAT enhancer binding protein-β (cEBP-β) (372, 375-376, 379-383). In our study, endometriosis sufferers demonstrated higher plasma levels of miR-155 than controls and it is possible the increased epidemiological risk of developing cancer seen in women with endometriosis, is conferred by this miRNA.

In summary, functional analyses of endometriosis-associated miRNAs and their predicted targets allowed identification of mRNA targets, biological and functional pathways and molecular networks that demonstrate concordance with our current knowledge of the cellular and molecular events associated with endometriosis. Additionally these analyses raise the possibility that plasma miRNAs may provoke some of the adverse health epiphenomenon associated with endometriosis. If this was validated in further study, therapeutic alteration of endometriosis-associated plasma miRNAs levels might have potential utility to reduce the risk of health sequelae from endometriotic disease.

A subset of the identified target transcripts was previously found to be differentially expressed in eutopic or ectopic endometrial tissue in endometriosis that has been shown by the intersection of miRNA data with 4 available wide genome profiling studies. The interaction between miRNAs under study and validated endometriosis associated genes did not show reciprocal relationship for all miRNA-mRNA pairs. Despite the fact that miRNAs negatively regulate the expression of their target genes, this observation does not necessarily show lack of functional relevance between the miRNA and mRNA in the datasets. Firstly, miRNAs act not

only through inducing target degradation, but also via translational repression, in which case only protein levels would be altered. Moreover, miRNAs were also described as being able to activate translation of their targets through binding to 3' UTR sites, additionally supporting that negative correlation of miRNA-target transcript abundance does not necessarily hold true (178). Finally, since each miRNA modulates translation of many genes simultaneously and vice versa can be subjected to multiple sources of regulation, the relationship between miRNAs and their targets is likely to involve complex mechanisms that might be particularly evident for the interactions between different compartments. Collectively, our results further highlight the putative role of miRNAs as contributors to endometriosis pathogenesis with their main biological functions linked to the disease.

The origin of endometriosis-associated circulating miRNAs remains unknown. Few miRNAs were dysregulated in both plasma and endometrial tissue comparisons and all of the miRNAs that were concordant in both types of study, were down-regulated in plasma. It is therefore unlikely that the direct excretion of miRNAs from endometriotic tissues into the circulation accounts for the presence of endometriosis-associated miRNAs. Endometriosis is associated with well documented disturbances in several immune cell lineages (27) and endothelial cell activation (24). It is plausible that systemic changes associated with endometriotic disease enhance miRNA secretion into the plasma from altered immune or endothelial cells. For example, the expression of miR-155 is greatly increased in CD4+ T cells (384) and has been shown to be induced by interferon-β (IFN-β), TNF-α, IL-1β and Toll-like receptor ligand in various immune cells (373, 385). The simultaneous regulation of many miRNAs may also reflect a modulation of miRNA processing, as it is known that cellular stressors can affect the expression of the enzyme Dicer that is essential for the biogenesis of mature miRNAs (386).

The discrepancy between the expression patterns between circulating miRNAs and miRNAs from endometrial sources could be attributed to miRNA turnover in the blood. Thus, it is still possible that some circulating miRNAs originate from the endometrium or endometriotic lesions. There is no information available on the bioavailability and half life of the circulating miRNAs at present to permit further evaluation of this possibility. Further experimental studies are necessary to explore the mechanism by which endometriosis affects tissue versus circulating miRNA levels.

4.4.4 Limitations

Our results should be interpreted with several limitations in mind. The first drawback of our study is its small sample size. The data presented need to be confirmed in larger clinical populations, particularly because the analysis of the disease severity and clinical subgroups (i.e. pain vs. infertility) is hampered by small numbers of patients available in these sub-cohorts. Secondly, we could not provide molecular insights into the cause of the dysregulation. It has been recently shown that exosomes and microparticles containing miRNAs can be released into the extracellular environment and then be internalized by other cells. Therefore, future studies will need to establish whether endometriosis-specific miRNAs in the bloodstream are contained in exosomes or microparticles, whether they can enter cells and, eventually, which cells and whether they have any biological action. Moreover, since the targets of miRNAs presented in the current study are just predictions based on mathematical algorithms, the validation of miRNA targets *in vitro* in a cellular system, is essential to evaluate the contribution of each miRNA to the overall modulation of gene expression. Overall, the mechanisms underlying the dysregulation, as well as the putative impact of the changes in circulating miRNAs levels in the physiology or pathophysiology of endometriosis remain to be determined.

4.5 Conclusions

In summary, this study provides an initial insight into the nature and function of circulating miRNAs associated with endometriotic disease. The results of the present study open two main areas of investigation. An improved understanding of the source, regulation and mechanism of action of circulating miRNAs may reveal new insights into the pathophysiology and systemic manifestations of endometriosis. Additionally, these miRNAs may prove to be biomarkers of disease or therapeutic targets for treatment of endometriosis. However, the results need to be confirmed and expanded in larger clinical populations. This will be particularly beneficial to determine if factors like disease severity and differences in clinical symptomatology impact on circulating miRNA profiles.

Chapter 5
Insight on the function of the dysregulated circulating miRNAs

5.1 Introduction

In the experiments reported in the previous chapter, we identified endometriosis specific miRNAs in plasma. Next, we wished to determine if these miRNAs are known to be associated with pathways linked to endometriosis and to understand how circulating miRNAs may contribute to the endometriotic disease process. For this purpose, we performed a review of seminal articles in the field with particular emphasis on the miRNA functional studies. In parallel, we retrieved the data compiled in IKB regarding the functional interactions between the dysregulated plasma miRNAs of interest.

Most of the circulating miRNAs identified by us have not been previously mentioned in the context of endometriosis. We therefore evaluated their role across different pathological conditions that share underlying pathophysiological mechanisms with endometriosis. In view of the inflammatory (1, 6, 28, 77, 387-388) nature of endometriosis and given that aspects of the process of endometriotic disease resemble that of metastatic lesions in malignancy (1, 6, 16), it was of particular interest to identify the involvement of the investigated miRNAs in aberrant immune function and in oncogenic processes.

5.2 Materials and methods

The current literature regarding each of the 12 miRNAs identified as significantly differentially expressed in plasma in endometriosis was reviewed, with particular emphasis on their involvement in human disease. The articles were identified using combinations of the following search terms in Entrez Pubmed and Google scholar: miRNA and disease; inflammation; immunity; endometriosis; reproductive tract; endocrine; E; P4 and the particular miRNAs (miR-155, miR-128, miR-362-3p, miR-662, miR-93*, miR-598, miR-224, miR-425, miR-23a, miR-574-3p, miR-29a*, miR-376a-5p). In addition, IPA software was used to access the experimentally validated data on the interrelationships of the miRNAs of interest.

5.3 Results

5.3.1 The experimentally validated functions and inter-relationships of the circulating miRNAs

Using the IPA software, we were able to gain additional insights into the validated functions and inter-relationships between the circulating miRNAs. Reproductive system disorder, cancer and endocrine disorder were the most significantly associated pathologies with these miRNAs. Experimentally validated relationships were identified for 6 out of the 12 dysregulated miRNAs

and these were integrated into a transcriptional network converging on connective tissue disorders, inflammatory disease and inflammatory response, as presented in Figure 5.1.

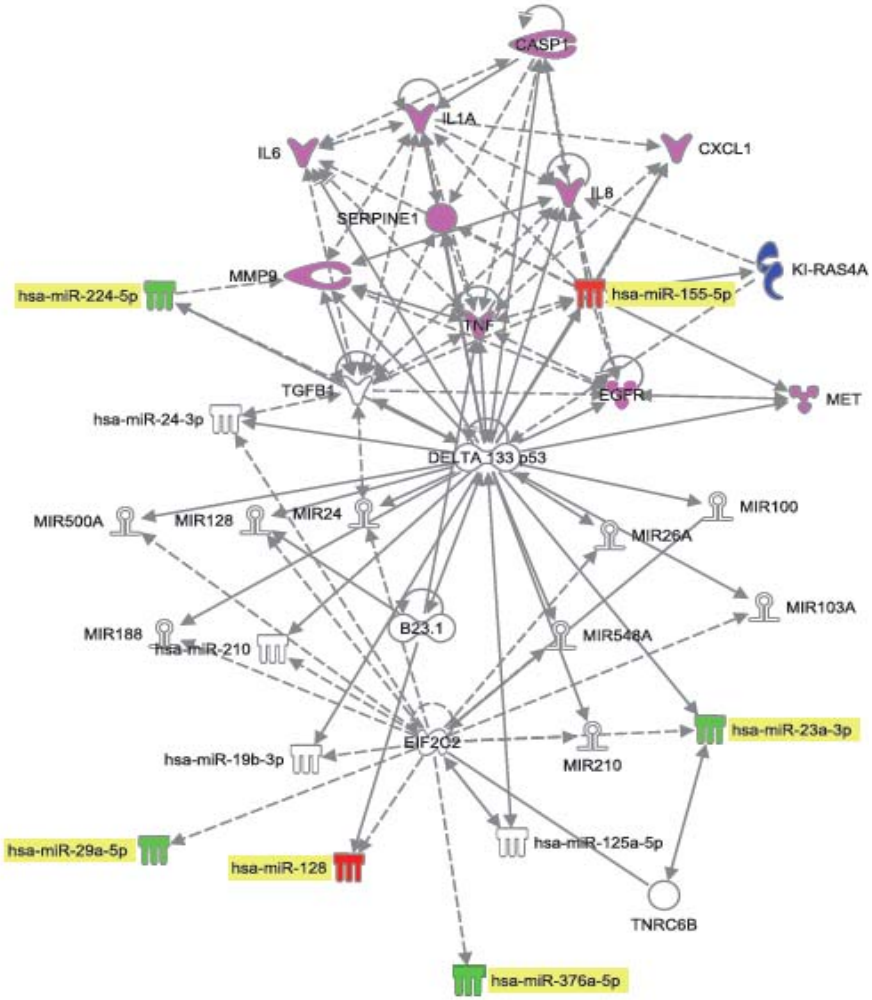


Figure 5.1 Example of the network enriched with 12 plasma miRNAs dysregulated in endometriosis, identified in IKB, IPA software. The network is associated with Connective tissue disorders, Inflammatory disease, Inflammatory response; score = 14.

Within the dataset, miR-155 appeared to be one of the most highly connected to inter- and intracellular molecular regulators with well characterised interactions. The regulatory loops of miR-155 involved TGF-β and TP-53 (tumour protein p-53) signalling as well as KRAS and MET proteins, which are at the heart of networks underpinning various malignancies. Additionally, miR-155 has been shown to interact with various inflammatory mediators, including TNF, ILs and MMPs. This suggests that miRNAs, particularly miR-155 might be a key factor linking inflammation, immunity and neoplasia with endometriosis. MiR-425-5p and miR-23a* were demonstrated to be involved in the same functional network with miR-155 via inflammatory or oncogenic mediators, suggesting complex interactive regulation of these miRNAs and the

possibility of joint mechanisms of action. Hypoxia-induced changes of Ago2, a critical component of RISC, have been linked with aberrant expression of miR-128, miR-23a, miR-376-5p and miR-29a-5p observed in cell lines (PASMC cells) (389). This suggests hypoxia as a possible regulatory mechanism underpinning miRNA expression as well as the involvement of these miRNAs in hypoxia-mediated events in endometriosis, particularly in aberrant angiogenesis and E2 signalling via possible modulation of the VEGF and HIF-1α, respectively (390-391).

5.3.2 Review of the role of circulating miRNAs in human disease

An outline of the pathological processes associated with the deregulated miRNAs, based on the literature survey, is summarised in Supplemental Material on the enclosed CD, Table 5S.

5.3.2.1 Up-regulated miRNAs

5.3.2.1.1 MiR-155

MiR-155 is one of the best described miRNAs and its aberrant expression has been reported in the context of inflammatory, auto-immune and malignant diseases (373, 384-385, 392-396). Contained within the non-coding B cell integration cluster (Bic) gene (397), miR-155 has been identified to regulate lymphoid development and immunity via modulation of the Toll-like receptor (TLR)-, IFN-β and TNF-α -mediated acquired and innate immune responses (373, 384-385, 398-401). In chronic inflammation, which is at the heart of autoimmune diseases, miR-155 exhibits increased expression in activated B, T and dendritic cells and is associated with altered functions of these cells. In particular, the expression of miR-155 is greatly increased in CD4+ T cells, which is associated with the skewing toward the T-helper 17 (Th17) cell lineage, along with promotion of expression of the cytokines required for inflammatory T cell development (384). Experimental mouse models show that miR-155 deficient mice are characterised by defective B and T cell immunity, and by substantial increase of IL-10, a cytokine associated with immunosuppressive activity against cell-mediated responses and by resistance to experimental induction of autoimmune conditions (384, 400-402). Further, miR-155 has been shown to be induced by IFN-β, TNF-α, IL-1β and TLR ligand in multiple immune cell lineages (373, 385, 393, 403-404). In an acute response, miR-155 is characterized as a component of the primary macrophage response to different types of inflammatory mediators (405).

Aberrant expression of miR-155 appears to mediate both pro- and anti-inflammatory activities, each of which could be involved in endometriosis (373, 384-385, 392-396).

The immune modulating effect of miR-155 is a complex multifaceted process that appears to involve many targets and pathways. For example, miR-155 has been demonstrated to down-regulate the transcription factor PU.1, associated with reduction of immunoglobulin1 (IgG1) class-switched differentiation in B cells and thus enhances production of IgG antibodies (402). On the other hand, activation-induced cytidine deaminase (AID), another *in vivo* validated target of miR-155, is required for high-affinity IgG antibody repertoire in antigen-activated B cells, in which case negative regulation by miR-155 provides an opposite result (406-407). In chronic inflammation, miR-155 might function to block the inhibitory impact of cytokines such as IL-4 and IFN-γ on CD4+ T cells through repression of c-Maf (408), and direct targeting of IFN-γ (409). Recent studies found that miR-155 targets Sma- and Mad-related protein 5 (SMAD5) in B cell lymphoma cells (410) and activates signal transducer and activator of transcription 3 (STAT3) in breast cancer cells (375), factors involved in TGF-β and IL-6 signalling with impact on Th cell lineage decisions.

MiR-155 has been linked with an inhibitory role on inflammatory cytokine production in response to microbial stimuli in activated antigen presenting dendritic cells via a negative feedback loop in the TLR/IL-1 cascade (399). Indeed, the silencing of miR-155 in dendritic cells has been associated with increased expression of the inflammation-related genes, including pro-inflammatory cytokines IL-1α, IL-1β, IL-6, IL-8, TNF-α and IL-23, the chemokines macrophage inflammatory protein (MIP)-1α/β, MIP-2α/β and MIP-3α and IL-1β-converting enzyme, caspase-1 [apoptosis related cysteine peptidase] (CASP-1) (399). Additionally, over expression of miR-155, induced by inflammatory cytokines, down-regulates the production of MMPs 1 and 3, the markers of the destructive inflammatory effects, suggesting a role for miR-155 in controlling tissue damage due to inflammation (393). Negative regulation of immune responses by miR-155 has been also observed through the targets of miR-155 that encode proteins involved in Lipopolysaccharide (LPS)/TNFα and TLR/IL-1 signalling, such as the Fas associated death domain protein (FADD), the IkB kinase epsilon (IKKέ) and the receptor interacting serine–threonine kinase1 (RIPK1) (373, 405). Moreover, miR-155 targets the transcription factor cEBP, which activates IL-6 gene expression (411).

In contrast, miR-155 can promote an inflammatory response via increased production of cytokines and chemokines, namely TNF-α, IL-6, IL-1β, IL-8, as demonstrated in CD14+ myeloid

cells in rheumatoid arthritis. Similarly, up-regulation of miR-155 provoked by exposure to LPS, enhances TNF-α production in mouse models (373, 405). The pro-inflammatory effect of miR-155 has been observed, for example, through a mechanism involving reduced Src homology 2-containing inositol phosphatase-1 (SHIP-1) that inhibits inflammation (392) and via suppression of suppressor of cytokine signalling-1 (SOCS1), a critical regulator of STAT signalling (412). It has been also hypothesized that miR-155 could increase cytokine levels directly by augmenting transcript stability through binding to their 3'UTR (373).

In view of the above, deregulation of miR-155 appears to be one of the central events in pathogenesis of endometriosis. Indeed, various cytokines associated with an aberrant immune response have been linked to the distant cell implantation (413), which also holds true for establishment of endometriotic lesions (1, 6) and thus might be linked to the pro-inflammatory features of miR-155. Moreover, a diminished immune response mediated by miR-155 and known to be associated with endometriosis would be expected to attenuate clearance of endometrial implants from the peritoneum and further promote the enhanced endometrial cell survival on the ectopic sites.

In line with these observations are the findings that miR-155 modulates expression of transcripts, namely IL-1α, CASP1, Serpine [plasminogen activator inhibitor type 1], chemokine (C-X-C motif) ligand-1 (CXCL1) and epidermal growth factor receptor (EGFR), that in addition to generation of inflammatory response are also involved in the genesis of many of the cellular events described in endometriosis (381, 399, 414). This includes chemotaxis, angiogenesis, axonal degeneration, cell migration, proliferation, apoptosis, production of PG-E2 as well as aberrant transcription factor NFkB, MAPK and TGF-β1 signalling (1, 6, 30, 371, 415-421). In turn, the regulation of miR-155 levels is hypothesized to be controlled, at least in part, by NFkB (405), MAPK and by c-Jun N-terminal kinase (JNK) pathways (404), all of which have been documented to have aberrant signalling in endometriosis (208, 371, 419-420, 422-425).

Multiple lines of evidence strongly support the significant role that miR-155 plays in tumour genesis and progression, acting predominantly as an oncogenic miRNA (oncomir) (373-378, 426). An oncogenic effect of miR-155 is demonstrated via translational inhibition of the tumour suppressor genes, including mRNAs coding for transcriptional regulatory proteins, receptors, kinases, and nuclear and DNA binding proteins. The direct targets of miR-155 in the context of cancer include FOXO3, Ras homolog gene family, member A (RHOA), melanocyte-stimulating hormone-2 (MSH2), MSH6, SOCS1, MET proto-oncogene, CK1a, SHIP and cEBP-β

(375-376, 379-383, 426). Regulatory feedback loop of miR-155 control involves aberrant TGF-β signalling, that is at the heart of various malignancies (427-429) . Expression of miR-155 is induced by TGF-β through Smad4 in animal models (376), whereas inhibition of TGF-β1 in siRNA transfected cervical carcinoma cell cultures (HeLa cells) results in decreased levels of miR-155 (429). MiR-155 interacts with tumour protein p-53 (TP-53) signalling, which is a key player in the cellular response to a variety of extra- and intracellular insults, such as DNA damage and oncogenic activation. TP-53 has been previously found to be over-expressed in endometriotic lesions (430), being linked to apoptosis resistance and enhanced survival of endometrial implants (431). The activation of TP-53 in lung cancer cell lines (H1299 cells) increases expression of miR-155 (432), whereas miR-155 attenuates TP-53 signalling via repression of TP-53-induced nuclear protein 1 (TP-53INP1), a proapoptotic gene downstream of TP-53 signalling (380).

5.3.2.1.2 MiR-128

MiR-128 is a brain-enriched miRNA that promotes neural differentiation (433) and has been found to be implicated in different cancers; for example, it is down-regulated in glioblastoma (434) however is up-regulated in endometrial cancers (435). Although the underlying mechanism of miR-128 involvement in tumourogenesis is just beginning to emerge, previous reports clearly demonstrate an anti-proliferative effect of this miRNA. For example, over-expression of miR-128 inhibits proliferation of glioma cells (436), reduces motility and invasiveness of neuroblastoma cells (437), negatively regulates prostate cancer cell invasion (438) and induces apoptosis pathways in embryonic kidney cells via modulation of the proapoptotic BCL2-associated X protein (Bax) (439). The expression of miR-128 in bone marrow of AML patients has been shown to be modulated by nucleoplasmin (NP1) (374), a nucleolar protein involved in tumour-associated chromosomal translocations and regulating the stability and activity of TP-53 (440), that in turn is involved in regulation of miR-155 and miR-224.

5.3.2.1.3 MiR-362-3p

MiR-362-3p, associated with mesenchymal stem cells (MSC) (441), which are also referred to as multipotent stromal cells, capable of self-renewing and differentiating into multiple cell lineages. MiR-362-3p has been found to be over-expressed in the peripheral blood in inflammatory bowel disease (442-443), and in serum of patients with enteroviral infections

(444). MiR-362-3p is deregulated in blood cells of melanoma patients (445), is over-expressed in plasma in glioblastoma (446) and is over-expressed in CD34+ cells from bone marrow in acute myeloid leukaemia (AML) (447). In human myeloid leukemic cell line, AML-193, miR-362-3p is found to be regulated by the cytokines involved in growth and differentiation of myeloid progenitors, namely IL-3 and granulocyte macrophage colony-stimulating factor (GM-CSF) (448). Further, miR-362-3p is down-regulated in cerebral ischemia and is involved in pathways associated with inflammation and apoptosis (449). MiR-362-3p is predicted to target sex determining region Y-related HMG box-17 (Sox17), an inhibitor of Wnt signalling pathway (450). Interestingly, the Wnt/ CTNNB1 pathway has been shown to be required for the E-induced proliferation of eutopic endometrium (451). Therefore, silencing of Sox17 by elevated expression of miR-362-3p may result in a similar up-regulation of the Wnt signalling pathway and promote growth of ectopic lesions in endometriosis.

5.3.2.1.4 MiR-662

While not much is known of miR-662, it was recently shown that it is transiently up-regulated in response to high doses of X-ray radiation in human fibroblasts (452) and was found to be more frequently gained in cell lines highly resistant to chemotherapeutics (453). MiR-662 is up-regulated in lupus nephritis (454), in lung cancer (455), in pituitary adenomas (456) and is down-regulated in cholangiocarcinoma (457) and ovarian cancer (458). The negative correlation observed between miR-662 and dual specificity phosphatase 5 (DUSP5) gene in monocytic cell lines, suggests their functional interaction and involvement of miR-662 in monocytic differentiation via MAPK signalling (459). Hence, miR-662 might have a similar effect in endometriosis.

*5.3.2.1.5 MiR-93**

Although there are no reports that directly address the functional role of miR-93* (miR-93-3p), this miRNA belongs to the miR-17 family, members of which, namely miR-17, miR-20a/b, miR-106a/b, and miR-93, act as oncogenes and immune-modulators within the functional cluster miR-17-92 (460-461). These miRNAs influence cell-cycle progression via targeting of CDKN1A, a potent negative regulator of the G1/S checkpoint (462-463). They also directly target proapoptotic gene BCL2L11/BIM, induce the angiogenesis inducer c-Myc oncogene and are involved in aberrant TGF-β signalling (460, 464). The established relevance of the above mechanisms in endometriosis supports the importance of miR-93-3p in the disease.

5.3.2.1.6 MiR-598

MiR-598 appears to be important for B lymphocyte development and is highly expressed in human peripheral blood mononuclear cells, especially activated B cells (465). A previous report showed that miR-598 expression is up-regulated in neural growth factor-treated rat pheochromocytoma PC12 cells (466), that might suggest its involvement in neo-innervation events that characterise endometriosis. MiR-598 has been found to be down-regulated in glioblastoma (467) and in hepatocellular carcinoma (HCC) (468), but up-regulated in chronic lymphocytic leukaemia (CLL) (469) and in colorectal carcinoma (470). The putative binding sites of the transcriptional factor Glial cells missing binding site (GBS), involved in cell cycle signalling, have been identified in the promotor of miR-598, indicating the involvement of GBS pathway in regulation of miR-598 expression (471). MiR-598 is an endogenous inhibitor of Sirtuin1 and the modulation of miR-598 affects senescence, proliferation, and differentiation in human adipose tissue-derived MSC (472). Finally, the observed binding of miR-598-3p to DNA-cytosine-5-methyltransferase-3 alpha (DNMT-3α), known to be involved in DNA replication, methylation and gene transcription, points toward a putative regulatory role of this miRNA in fundamental cell processes (473).

5.3.2.2 Down-regulated miRNAs

5.3.2.2.1 MiR-224

MiR-224 is highly over-expressed in prostate, bladder and in HCC, but is down-regulated in ovarian epithelial and lung cancer (474-475). MiR-224 directly targets cell division cycle 42 protein (CDC42) (476) that contributes to G1/S phase progression through p70 S6 kinase-mediated induction of cyclin-E expression, indicating the possible positive role of decreased miR-224 on cell development via eliminating translational suppression on G1 arrest (477). Further, down-regulation of miR-224 in endometriosis attenuates other functions attributed to this miRNA, such as inhibition of cell proliferation (478), promotion of apoptosis by suppressing the apoptosis inhibitor API-6 (479), mediation of TGF-β1-induced granulosa cell proliferation via targeting SMAD4 and promotion of E2 release from granulosa cells via CYP19A1 (480). Moreover, the loss of function study demonstrated that silencing of miR-224 is associated with decreased expression of MMP9 (481), suggesting a pro-inflammatory effect of this miRNA. Similarly to miR-155, the expression of miR-224 is up-regulated by factors

involved in inflammation, namely LPS, TNF-α and NFkB pathway as well as the oncogene TP-53 (432, 482-483).

5.3.2.2.2 MiR-425

Over-expression of miR-425 has been described in several human cancer cells (484-485), supporting involvement of this miRNA in cell proliferation and oncogenic activity. MiR-425 was identified activated in human sarcoma in association with RAS-signalling (486) and down-regulated in a subset of human primary medulloblastoma in association with aberrant Hedgehog signalling (487). MiR-425 has recently been identified as over-expressed in MSC (441) and was demonstrated to promote osteoblast differentiation of MSC via targeting of Sequestome1 (SQSTM1) involved in NFkB regulation (488). This suggests a possible role of miR-425 in the molecular mechanisms underlying cell self-renewal, differentiation, replicative senescence, and wound healing. Transfection of bone marrow CD133+ cells with miR-425 led to their significantly decreased proliferation and differentiation, likely via repression of the ubiquitin proteasome system (UPS). MiR-425 was found to be decreased in the CD133+ population in glioblastoma multiforme (GBM) where it inhibited neurosphere formation through SMAD3 and SMAD4 activation in GBM stem cells (489). Thus, the down-regulation of miR-425 in endometriosis might enhance proliferation and neurogenesis. Recently, it has been shown that miR-425 is regulated by the transcriptional repressor RE1-silencing transcription factor (REST), which is essential to prevent precocious neuronal differentiation and self-renewal of neural stem cells (490). MiR-425 is over-expressed in Treg cells, required for the maintenance of immune homeostasis, including anti-tumour immune response (385). MiR-425 is involved in human NK cell activation through IL-2, IL-15 and IL-21 stimulation (491). MiR-425 is up-regulated in cirrhosis and in human HCC cell lines (483), suggesting its role in liver pathology and disease progression. MiR-425 has been predicted to target methyl guanine methyl transferase (MGMT) (492), an evolutionarily conserved DNA repair enzyme (493) that restores guanine nucleotides that are alkylated at the O6 position to the native, undamaged form. The under-expression of miR-425 observed in plasma in endometriosis can lead to increased MGMT protein level, similar to that observed in normal immune cells that infiltrate the tumour mass (494). Additional predicted targets of miR-425 include DICER1 and SMAD2 (495). SMAD is involved in the regulation of DROSHA, another key player in small RNA processing (496), indicating that aberrant expression of miR-425 might have important effects in the biogenesis of small RNAs.

5.3.2.2.3 MiR-23a

MiR-23a is linked to cell cycle, proliferation, differentiation, haematopoiesis, cardiac and inflammatory disease (442-443, 497). Similarly to other miRNAs, miR-23a is involved in progression and development of human cancers in cell-dependent manner, being down-regulated in some types of cancers (prostate, lymphoma, leukaemia) and up-regulated in others (colorectal, bladder, gastric, breast, pancreatic cancer, glioblastoma, and HCC) (498). MiR-23a is one of the important players in the reproductive tract disorders (499). In human granulosa cells, miR-23a has been associated with inhibition of testosterone, cell proliferation and apoptosis (478). MiR-23a was found to be over-expressed in plasma of women with premature ovarian failure and appeared to have regulatory effects on proliferation and apoptosis by targeting X-linked inhibitor of apoptosis protein (XIAP), Phosphatase and tensin homolog (PTEN), and CASP-7 in human ovarian granulosa cells (500). Some other authors have demonstrated down-regulation of mir-23a in ectopic compared to eutopic endometrium in endometriosis (209, 233), while others observed up-regulation in ovarian endometriomas compared to eutopic endometrium in controls (207). While the expression of miR-23a was further down-regulated by E2 and P4 in endometrial stromal cells, miR-23a appear to play an important role in steroidogenesis via targeting genes that play a central role in this process, namely StAR, CYP19A1 and COX-2 (233). Even though multiple mechanisms of miR-23a activity have been described, the particular relevance to the molecular events in endometriosis can be also observed via the involvement of this miRNA in TNF-α and TGF-β1 signalling. The down-regulation of miR-23a in endothelial cells has been shown to increase TNF-α-induced endothelial cell apoptosis through regulation of the CASP-7 and serine/threonine kinase4 pathways, suggesting protective effect of miR-23a against TNF-α activity (501). Additionally, miR-23a was found to be regulated by TGF-β1 in a SMAD-dependent manner in lung adenocarcinoma cells and in contrast was demonstrated to regulate TGF-β-induced epithelial-mesenchymal transition by targeting E-cadherin (502).

5.3.2.2.4 MiR-574-3p

Aberrant expression of miR-574-3p has been reported in association with several malignancies and autoimmune diseases. MiR-574-3p is down-regulated in bladder (503), lung (504) and gastric cancer (505), however it is up-regulated in urine of prostate cancer patients (506), in lymphocytes in B-cell lymphoma (507), in serum of HCC (508) and in salivary glands in Sjogren's syndrome (509). A tumour suppressor role of miR-574-3p has been demonstrated in

functional studies where miR-574-3p over-expression inhibited the proliferation, migration and invasion ability of gastric cancer cells (505). One of the proposed mechanisms involves direct targeting of mesoderm development candidate 1 (MESDC1), which stimulates proliferation and invasion ability in bladder cancer cells (503). Hence, the down-regulation of miR-574-3p observed in plasma in the current study may be associated with development and progression of the endometriotic lesions. Although the regulatory mechanism of miR-574-3p is largely unknown, miR-574-3p appears to be regulated by CTNNB1/TCF4 pathway, which is embedded in the Wnt-signalling network (510), shown to be deregulated in endometriosis (511-512). A recent functional study demonstrated that knock-down of TAR-DNA-binding protein-43 (TDP-43) caused significant reduction of miR-574-3p levels in HEK293T-derived cell lines (513). TDP-43 is homologous to the heterogeneous ribonucleoproteins, which are involved in RNA processing, and its abnormal function is a key feature of the neurodegenerative diseases and decreased axonal growth (513-514). Considering the well documented state of aberrant innervation in endometriosis (515), it is possible to assume that this, at least in part, is linked with alterations in TDP-43 metabolism, that consequently leads to a decrease in miR-574-3p expression, which further contributes to the pathogenesis of endometriosis.

*5.3.2.2.5 MiR-29a**

MiR-29a* (miR-29a-5p) belongs to the miR-29 family, members of which share a common seed region sequence and are predicted to target largely overlapping sets of genes. Even though there are no reports on the functional relevance of miR-29a*, the members of the miR-29 family are important regulators of human diseases (516-519). Mir 29s have been shown to have strong anti-fibrotic and proapoptotic effects, along with involvement in cell differentiation and immune regulation (519). For example, miR-29 is down-regulated in activated immune cells and controls innate and adaptive immune responses by directly targeting INF-γ (520) as well as T cell polarization, and B cell oncogenic transformation (521). The involvement of miR-29 in malignancy has been linked to its ability to directly target the series of oncogenes T cell leukaemia/lymphoma 1 (TCL1) and myeloid leukaemia cell differentiation protein (MCL1) as well as to cause epigenetic changes characteristic of malignancies. Members of the miR-29 family have been shown to be down-regulated in aggressive and invasive forms of cancer, namely AML, CLL, breast and lung cancer, HCC and cholangiocarcinoma (522). The miR-29 family is known to target and down-regulate DNMT3A,

DNMT3B, and DNMT1 (523), in which case down-regulation of miR-29 is linked with distortion of the DNA methylation pattern with consecutive effect on genes involved in tumourogenesis and apoptosis (524). TGF-β1 signalling, a key driver of fibrosis, as well as the elevated NFkB levels triggers silencing of the miR-29 family (518, 525-526). Therefore it is tempting to assume that down-regulation of plasma miR-29a* observed in the current study might be directly associated with aberrant TGF-β1 and NFkB signalling in endometriosis.

5.3.2.2.6 MiR-376a-5p

MiR-376a-5p has been deregulated in a wide range of the diseases, including traumatic brain injury, Alzheimer's disease, squamous oesophageal carcinoma, pancreatic carcinoma, endometrial carcino-sarcomas, lung cancer, breast cancer, pleomorphic adenomas of the salivary gland, primary pigmented nodular adrenocortical disease and medulloblastoma (527). MiR-376a-5p is involved in signal transduction, long-term potentiation, as well as negative regulation of microtubule depolymerisation and microfilament motor activity.

5.4 Conclusions

In this chapter, we summarised the existing evidence on involvement of endometriosis-specific plasma miRNAs in human disease. Aberrant expression of these miRNAs has been described in context of inflammatory, auto-immune and malignant diseases. The functional associations and regulatory mechanisms for most of the miRNAs under study is just beginning to emerge, however it is evident that TP-53, TGF-β, MAPK, NFkB and Wnt signalling are likely to modulate the expression of these miRNAs. Notably, for most of the investigated miRNAs, the direction of deregulation tends to vary in different types of malignancy, suggesting their ability to act as oncogene and as tumour-suppressors, presumably in a tissue- or disease-specific manner. A similar pattern has been observed in the inflammatory response, being particularly evident for miR-155. Hence, the pro- and anti-inflammatory as well as oncogenic responses are finely regulated by miRNAs in different and still not entirely understood ways.

Altogether, these data point out that aberrantly expressed plasma miRNAs are involved in numerous biological processes that have been linked to systemic and local tissue effects in endometriosis. Moreover, miRNAs are closely integrated into regulatory networks and their function depends on complex homeostasis of various miRNAs and their targets.

Chapter 6
MiRNA expression profile in eutopic endometrium in endometriosis

6.1 Introduction

Current models of endometriosis assume that endometriosis is characterised by intrinsic endometrial abnormalities that also give rise to the impaired implantation and infertility associated with the disease (1, 30). However, the mechanism by which the presence of disease alters the eutopic endometrium is not established. Moreover, it remains unclear whether alterations of the eutopic endometrium are the consequence of endometriosis or can be also causally related to establishment of the disease.

We have identified endometriosis-specific miRNA signatures in plasma (Chapter 4), which raises provocative questions regarding their origin and potential biological role in the pathogenesis of the disease. While the mechanism for how miRNAs reach the systemic circulation is still unknown, we hypothesised that miRNAs are released from aberrant eutopic endometrial tissue. Another possibility is that circulating miRNAs could directly influence endometrial cells in both ectopic lesions and eutopic endometrium. Thus, characterisation of the miRNA profile in eutopic endometrium can promote our understanding of the origin of circulating miRNAs and of the molecular events involved in endometriosis. Previously, only one group has specifically looked at miRNA expression in eutopic endometrium in endometriosis, in comparison to women with leiomyoma (206). Even though no leiomyoma associated miRNAs (528) were found to be dysregulated in the study, complex interrelationships between miRNAs make it difficult to completely exclude that the study findings were not confounded by leiomyoma.

We wanted to explore the correlation between dysregulated miRNAs in eutopic endometrium and those identified in plasma in endometriosis (Chapter 4). An additional aim was to determine if miRNA signatures in eutopic endometrium can discriminate between symptomatic women with and without endometriosis and thus have potential as an endometrial biopsy-based test to diagnose endometriosis.

Two main confounders were addressed by the study. Firstly, several previous reports suggested menstrual cycle dependent differences in miRNA (235) and gene expression (364) in eutopic cycling endometrium in healthy subsets as well as in eutopic endometrium from women with surgically proven endometriosis (96). In order to reduce the confounding effect of cycle stage and given that transcriptome of the secretory stage of the cycle is the best characterised in the literature, we focused on secretory endometrium. Additionally, the

selection of the control group is important as we assumed that endometrium of endometriosis-free women with pain or infertility might differ from that of disease-free women without any symptoms. We conducted large-scale miRNA profiling using the Affymetrix microarray platform using secretory endometrium obtained from symptomatic (pain ± infertility) women with a surgically proven diagnosis of endometriosis and from the groups of symptomatic and asymptomatic controls with surgically confirmed normal pelvis. Each comparative analysis was performed separately for each control group as well as for the combination of both.

6.2 Materials and Methods

6.2.1 Study Participants

Patients undergoing surgery for suspected endometriosis (pain ± infertility) or benign disorders were approached for participation. The samples were collected in the period from March 2010 to April 2011. The inclusion criteria were: women 18-45 years of age with regular menstrual cycles (21-35 days), and a willingness to participate in the study. The exclusion criteria were: breast feeding, pregnancy, cancer or any other systemic medical condition, including acute infection at any site from 2 weeks prior to sample collection; known uterine pathology, and any type of medical treatment at least 3 months preceding surgery. Menstrual cycle phase was determined from the history of the last menstrual period and confirmed by a serum hormonal profile and by histological evaluation of the tissue according to Noyes criteria, which was performed by an experienced pathologist (529). Endometrial tissue was obtained from the 17 participants undergoing laparoscopy for diagnosis and treatment of pelvic pain ± infertility and from 11 asymptomatic participants undergoing laparoscopy for benign disorders. A total of 13 patients were in the secretory phase of the cycle and were included in the study.

6.2.2 Tissue collection, RNA extraction and sample quality control

The methodology used for tissue collection, RNA extraction and assessment of the quality and quantity of the isolated miRNA samples are presented in Chapter 3. The characteristics of the samples are presented in Table 6-1.

Table 6-1 Laboratory characteristics of the samples

Sample code	nanodrop readings pre Dnase tx			Dilution	nanodrop readings post Dnase tx			Gel quality	Bioanalyser, RIN
	A260/280 nm	A260/230 nm	Concentration ng/ul		A260/280 nm	A260/230 nm	Concentration ng/ul		
P1	2.02	1.57	917.75	X 2	1.96	1.57	250.24	good	6.2
P2	2.02	1.67	1660.23	X3	1.98	1.57	181.02	good	3.4
P3	1.94	1.62	423.25		1.96	1.56	169.24	good	5.7
P4	1.91	1.48	318.48		1.99	1.54	209.17	good	8.1
P5	1.99	1.26	235.03		1.93	1.25	175.56	good	8.2
Asympt ctrl 1	2.05	2.02	1144.54	X 2	1.98	1.79	289.3	good	4.1
Asympt ctrl 2	1.89	1.65	600.71		1.97	1.67	276.27	good	2.4
Asympt ctrl 3	1.8	1.94	3075.38	X 5	1.95	1.86	405.23	good	5.1
Asymp ctrl 4	2.03	1.46	1846.04	X5	2.02	1.45	181.97	good	3
Sympt ctrl 1	1.98	2.01	1072.59	X 2	1.97	1.82	230.88	good	8.3
Sympt ctrl 2	2.04	1.51	919.44	X3	2	1.57	196.4	good	6.6
Sympt ctrl 3	2.06	2.03	1105.53	X6	1.99	1.74	163.7	good	2.9
Sympt ctrl 4	1.92	1.36	162.38		1.9	1.32	124.06	poor	2.6

Despite adequate RNA integrity demonstrated by agarose gel electrophoresis in all the samples, some had low RIN, suggesting degraded RNA. We could not explain the discrepancy between these methods. It was unclear how this finding (the combination of low RIN and appropriate charge and size separation on gel) could influence miRNA profiling. Both gel electrophoresis and Bioanalyzer assessment did not directly assess the quality of miRNA in the sample. Given the higher stability of miRNA compared to mRNA, it is possible that miRNAs in a sample are preserved with good quality in a presence of degraded total RNA. Additionally, spectrophotometric assessment revealed relatively low absorbance ratio A260/A230, suggesting contamination of organic compounds. Repeat precipitation with isopropanol followed by a wash with ethanol improved the result but it remained below the expected value of 2.0. The consequence of this on the performance of the hybridisation array was unclear. Thus, we undertook additional sample quality testing by performing qRT-PCR, assuming that the factors responsible for RT-PCR inhibition are likely to interfere with performance of hybridisation. We selected to test the samples with low RIN and 2 samples with high RIN for miRNA expression by using TaqMan miRNA assays, aiming to exclude the samples with poor PCR performance from the microarray experiment. The selected miRNAs for testing with qRT-PCR included miR-222, that was reported as abundantly expressed miRNA

in luteal endometrium (235) and small RNA mammalian U6, known to be readily present in a range of tissues and considered by many as a useful endogenous control. Overall, the assays performed well in all the samples with SD of the technical replicates < 0.5 Cq. No direct correlation was observed between the Cq values and RIN (Figure 6.1), thus all the samples were included in the microarray experiment.

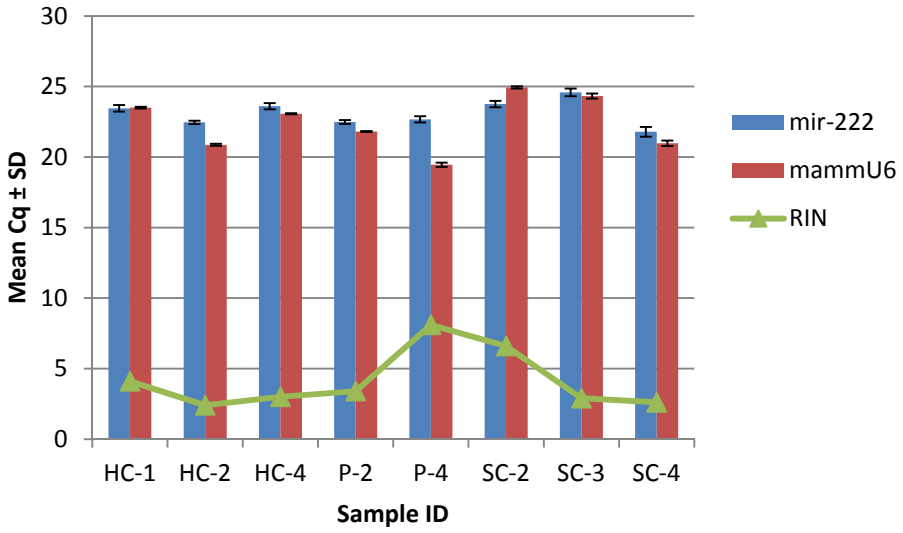


Figure 6.1 The relationship between endometrial miRNA levels measured by qRT-PCR and RIN: expression of miRNAs measured by qRT-PCR in the samples with low and high RIN. No direct correlation was observed between the quality of total RNA measured by RIN (Bioanalyzer) and the expression levels of miRNAs (miR-222 and Mammalian U6) expressed as Cq values (qRT-PCR).

6.2.3 MiRNA profiling

For miRNA expression screening in endometrial tissue, we used Affymetrix hybridisation miRNA microarray, as presented in Chapter 3.

6.3 Results

6.3.1 Study Participants

A total of 13 participants met the inclusion criteria and were included in the study. Five women were diagnosed with endometriosis (4 with minimal/mild, rASRM stage I-II and 1 with moderate, rASRM stage III), and 8 were surgically confirmed non endometriosis controls (4 women had the symptoms of pelvic pain and 4 were pain -free who had a laparoscopy for surgical sterilisation, n = 3 or for uterine prolapse, n = 1). The patients age ranged from 27 to 42 years, all participants had a regular menstrual cycle, were documented not to be pregnant,

and not to have had hormone treatment for at least 3 months before surgery. Age, BMI and parity were comparable between the groups. Endometrial biopsies of the participants were dated as mid to late secretory. The hormone profile of each patient was consistent with ovulatory cycle. Even though women with endometriosis had lower levels of P4, such difference was of little clinical and statistical significance. The patients' characteristics are presented in Table 6-2.

Table 6-2 Characteristics of the study population

	Endometriosis	Symptomatic controls	Asymptomatic controls	p-value
Age, years (Mean ± SD)	36 ± 5.6	35.3 ± 7.1	35.8 ± 5.6	NS
BMI, kg/m² (Mean ± SD)	25.5 ± 3.4	25.7 ± 7.1	25.2 ± 5.1	NS
Parity:				
0	2	2		
1	1	1		
2	2	1	2	
3			2	
Progesterone, nmol/L Median [range]	15 [12-40]	25 [11-38]	20 [15-54]	NS
Indication for surgery:				
Pain	2	2		
Pain + infertility	3	2		
Surgical sterilisation			3	
Uterine prolapse			1	
rASRM stage of endometriosis:				
I/II	3	-	-	
III	1	-	-	

6.3.2 Summary on QC of the microarray data

The distributions of raw probe level data were similar and typical of the distributions of other Affy miRNA chips, Figure 6.2A. In addition, when all chips were correlated against one another using all probe-level data, no chips displayed an outlier pattern, Figure 6.2B. The log intensities of the normalised and un-normalised data are presented in Figure 6.2C. There were no significant differences in the results when the data was normalised using 2 different software packages (Partek Genomics Suite and Affy R package), Figure 6.2D. The normalised data for the same chip (sample) performed by different algorithms was closer than the data from 2 different chips normalised by the same algorithm, Figure 6.2E. Overall the above observations suggested that the data was of good technical quality. Out of the total of 7822 probe sets from the different species, the array contained 1500 human only probe sets. Of these, 847 miRNAs were detected in endometrial tissue and were selected for the analysis.

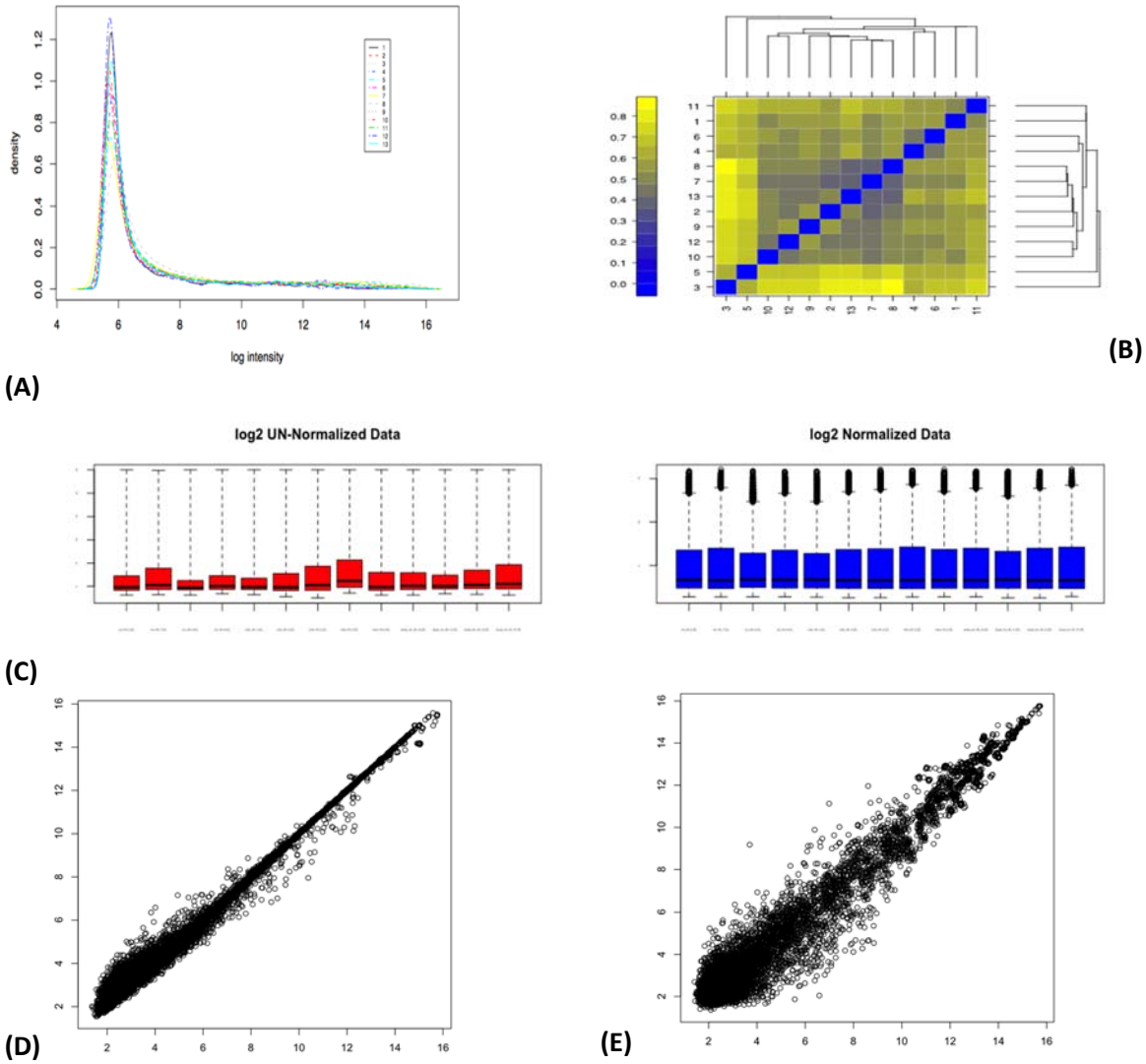


Figure 6.2 Diagnostic plots - exploratory analysis of the microarray data, Quality Control. Panel A represent the density plots of distribution of the signal intensity between the arrays, raw data. All the arrays demonstrated similar unimodal distribution, indicative for good quality with similar background intensities and absence of spatial artefacts. Panel B represent the Heat map, demonstrating the distance between the arrays when all probe data of each array is plotted vs. the others. The data exhibit reasonable correlation with no particular outliers. Panel C represent distribution of the array intensities before and after normalisation, one box plot per array. After normalisation all the boxes had similar spread, centring at the same level, suggestive of comparable quality homogenous data and absence of outliers. Panels D and E demonstrate correlation of the normalised data between the 2 arrays (the normalised log_2 intensities of each array included in comparison is plotted on the separate axis (x and y): Panel D shows correlation between the same chip normalised by 2 softwares, whereas Panel E shows correlation between 2 chips normalised by the same technique, both are suggestive of good correlation within the data.

6.3.3 Differentially expressed miRNAs

Two computational methods (ANOVA and LIMMA) were used to estimate differential expression of miRNAs between the groups and miRNAs that were common to both methods were added to the final miRNA list. Only 2 miRNAs were significantly differentially expressed between the endometriosis and control groups ($p < 0.01$), of which one miRNA was up-regulated (miR-551a) and 1 miRNA was down-regulated (miR-221). An additional 13 miRNAs demonstrated trend for statistical significance ($0.01 < p < 0.05$), (Table 6-3 and Figure 6.3). None of the miRNAs were significantly differentially expressed when multiple testing correction was applied, thus the likelihood of "false discovery" was high.

Table 6-3 Dysregulated miRNAs in eutopic endometrium in endometriosis (n = 5) vs. overall disease-free controls (n = 8)*

Endometriosis vs. All controls							
ID	**FC[1] LIMMA**	**FC[1] ANOVA**	**p-value[2] LIMMA**	**p-value[2] ANOVA**	**adj. p-value[3] LIMMA/ANOVA**	**t[4] LIMMA**	**t[4] ANOVA**
miR-551a	**1.83**	**1.77**	**0.002**	**0.003**	**0.76**	**3.82**	**3.98**
miR-221	**-1.44**	**-1.43**	**0.007**	**0.003**	**0.94**	**-3.18**	**-3.82**
miR-335	-2.70	-2.54	0.002	0.012	0.76	-3.89	-3.04
miR-615-5p	1.68	1.38	0.003	0.012	0.94	3.50	3.04
miR-223	2.18	2.13	0.010	0.027	0.94	2.99	2.59
miR-141	3.78	3.67	0.011	0.030	0.94	2.93	2.52
miR-214*	-1.62	-1.67	0.014	0.021	0.94	-2.80	-2.74
miR-513a-5p	-1.64	-1.65	0.023	0.006	0.94	-2.55	-3.47
miR-629*	1.73	1.80	0.024	0.024	0.94	2.53	2.65
miR-302c	1.38	1.29	0.024	0.047	0.94	2.52	2.27
miR-134	-1.39	-1.38	0.025	0.024	0.94	-2.50	-2.67
miR-409-3p	-1.44	-1.45	0.029	0.032	0.94	-2.43	-2.48
miR-99a*	2.09	2.24	0.030	0.043	0.94	2.41	2.32
miR-661	1.43	1.43	0.035	0.013	0.94	2.33	3.03
miR-1287	-2.08	-2.31	0.040	0.020	0.94	-2.26	-2.77

* Summary of the miRNAs that overlap in both analyses, LIMMA and ANOVA; the level of significance was determined as $p < 0.01$, the trend for significance was determined as $0.01 < p < 0.05$;

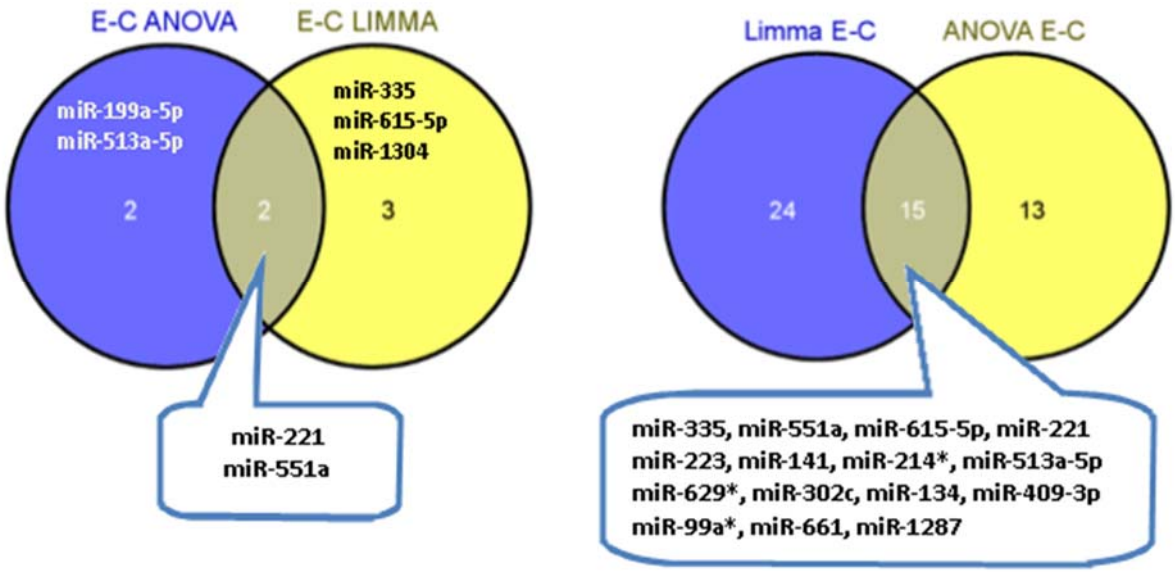
[1] **FC** = Fold change = Expression E/Expression C. Negative FC means down-regulated miRNAs in endometriosis vs. controls, positive FC – up-regulated miRNAs;

[2] **p-value** – probability of significant difference between the groups, not corrected for multiple testing;

[3] **adj. p-value** – associated p-value after adjustment for multiple testing by Benjamini and Hochberg's method;

[4] **t** = t-statistics;

miRNAs significant by both methods ($p < 0.01$) are presented in bold.



(A) **(B)**

Figure 6.3 MiRNAs dysregulated in eutopic endometrium in endometriosis demonstrated with 2 different analyses, ANOVA and LIMMA. Panel A represents significantly dysregulated miRNAs, $p < 0.01$; Panel B represents miRNAs exhibiting significance and trend for significance, $p < 0.05$.

Heat maps for the top ranked miRNAs showed segregation of the samples according to the endometriosis and control status, with the exception for one endometriosis, and one control from each subgroup, as presented in Figure 6.4. Considering separate clustering of the control subgroups, we performed separate analyses by comparing samples of endometriosis with each type of controls. Two miRNAs were significantly differentially expressed between endometriosis and symptomatic controls (miR-551a, miR-148a*) and 2 miRNAs between endometriosis and asymptomatic controls (miR-551a, miR-412), whereas 21 and 17 miRNAs exhibited a trend to significance between the subgroups, respectively (Table 6-4, Table 6-5). Little concordance was demonstrated between the lists of dysregulated miRNAs obtained when endometriosis was compared separately to symptomatic or asymptomatic controls.

A direct comparison of the eutopic endometrium miRNA profile from symptomatic vs. asymptomatic controls, revealed 4 significantly differentially expressed miRNAs of which 3 were up-regulated (miR-412, miR-934, miR-148a*) and 1 down-regulated (miR-1256) in symptomatic controls and additional 27 miRNAs that differed with a trend for significance (Table 6-6).

Taken together, our results indicate that eutopic endometrium in endometriosis has a distinctive miRNA profile. However, eutopic endometrium in women without endometriosis

represents a heterogeneous entity, exhibiting varying miRNA expression patterns in women with pelvic pain and in symptom-free women.

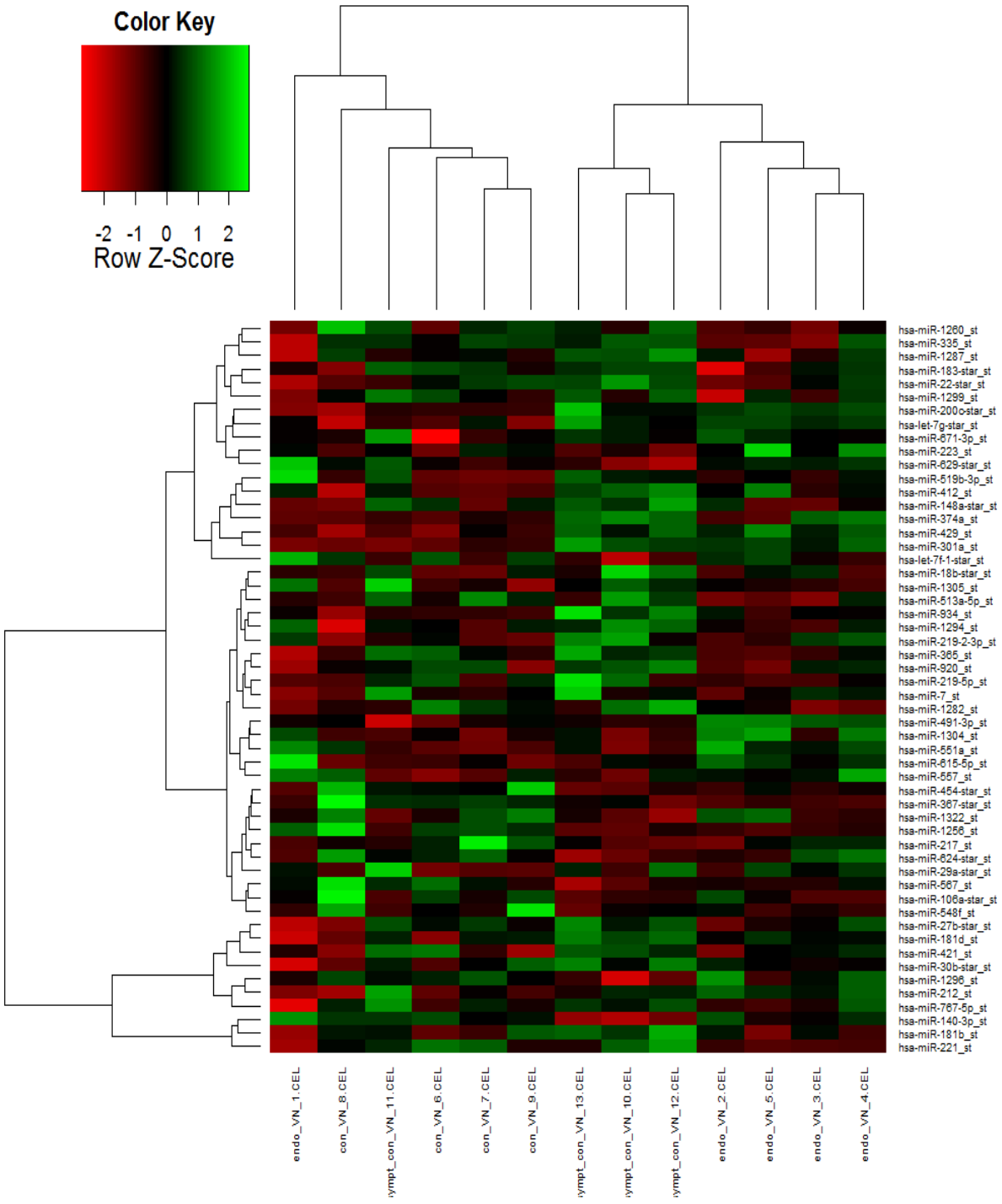


Figure 6.4 Heat map analysis - the top ranked endometrial miRNAs dysregulated in endometriosis. The ordering of the miRNAs is given by clustering based on the similarity of the miRNA expression patterns.

Table 6-4 Dysregulated miRNAs in eutopic endometrium in endometriosis (n = 5) vs. symptomatic controls (n = 4)*

ID	FC[1] LIMMA	FC[1] ANOVA	p-value[2] LIMMA	p-value[2] ANOVA	adj.p-value[3] LIMMA/ ANOVA	t[4] LIMMA	t[4] ANOVA
miR-551a	**1.83**	**1.78**	**0.008**	**0.007**	**0.99**	**3.13**	**3.41**
miR-148a*	**-2.83**	**-2.51**	**0.003**	**0.008**	**0.99**	**-3.65**	**-3.33**
miR-365	-1.81	-1.61	0.003	0.018	0.99	-3.55	-2.82
miR-335	-2.89	-2.81	0.004	0.017	0.99	-3.43	-2.87
let-7f-1*	2.58	2.21	0.013	0.039	0.99	2.86	2.38
miR-221	-1.47	-1.46	0.016	0.006	0.99	-2.77	-3.46
miR-140-3p	1.68	1.66	0.016	0.018	0.99	2.75	2.82
miR-223	2.35	2.44	0.018	0.027	0.99	2.71	2.60
miR-181b	-1.59	-1.61	0.020	0.016	0.99	-2.65	-2.89
miR-767-5p	-2.16	-2.08	0.022	0.043	0.99	-2.59	-2.31
miR-629*	1.94	2.03	0.023	0.022	0.99	2.57	2.71
miR-513a-5p	-1.81	-1.74	0.023	0.009	0.99	-2.56	-3.26
miR-1287	-2.62	-2.98	0.024	0.012	0.99	-2.55	-3.07
miR-615-5p	1.57	1.37	0.025	0.029	0.99	2.53	2.55
miR-1299	-3.08	-3.02	0.025	0.038	0.99	-2.53	-2.39
miR-27b*	-1.59	-1.60	0.032	0.041	0.99	-2.39	-2.35
miR-934	-1.62	-1.64	0.042	0.034	0.99	-2.25	-2.45
miR-7	-1.58	-1.57	0.042	0.035	0.99	-2.25	-2.43
miR-183*	-1.87	-1.79	0.042	0.032	0.99	-2.25	-2.50

* Summary of miRNAs that overlap in both analyses, LIMMA and ANOVA; the level of significance was determined as $p < 0.01$, the trend for significance was determined as $0.01 < p < 0.05$;

[1] **FC** = Fold change = Expression E/Expression C. Negative FC means down-regulated miRNAs in endometriosis vs. controls, positive FC – up-regulated miRNAs;

[2] **p-value** – probability of significant difference between the groups, not corrected for multiple testing;

[3] **adj. p-value** – associated p-value after adjustment for multiple testing by Benjamini and Hochberg's method;

[4] **t** = t-statistics;

miRNAs significant by both methods ($p < 0.01$) are presented in bold.

Table 6-5 Dysregulated miRNAs in eutopic endometrium in endometriosis (n = 5) vs. asymptomatic controls (n = 4)*

ID	FC[1] LIMMA	FC[1] ANOVA	p-value[2] LIMMA	p-value[2] ANOVA	adj.p-value[3] LIMMA/ ANOVA	t[4] LIMMA	t[4] ANOVA
miR-412	**1.86**	**1.90**	**0.004**	**0.000**	**0.93**	**3.45**	**5.06**
miR-551a	**1.83**	**1.76**	**0.008**	**0.007**	**0.93**	**3.14**	**3.35**
miR-615-5p	**1.80**	**1.39**	**0.005**	**0.026**	**0.93**	**3.31**	**2.62**
miR-367*	-1.67	-1.51	0.007	0.013	0.93	-3.22	-3.01
let-7g*	3.13	2.47	0.007	0.026	0.93	3.16	2.61
let-7i*	2.69	2.47	0.008	0.040	0.93	3.11	2.36
miR-335	-2.52	-2.30	0.010	0.044	0.93	-2.98	-2.31
miR-564	-2.46	-2.11	0.012	0.041	0.93	-2.92	-2.34
miR-429	4.23	3.85	0.012	0.041	0.93	2.90	2.35
miR-135b*	1.50	1.41	0.014	0.023	0.93	2.83	2.69
miR-1256	-1.63	-1.74	0.015	0.002	0.93	-2.78	-4.10
miR-141	4.40	4.34	0.018	0.036	0.93	2.71	2.42
miR-454*	-1.56	-1.76	0.019	0.003	0.93	-2.67	-3.94
miR-214*	-1.71	-1.76	0.023	0.028	0.93	-2.58	-2.57
miR-302c*	-1.92	-1.87	0.023	0.029	0.93	-2.57	-2.56
miR-548f	-1.81	-1.60	0.024	0.043	0.93	-2.55	-2.32
miR-874	1.67	1.82	0.028	0.048	0.93	2.46	2.25
miR-221	-1.41	-1.40	0.029	0.013	0.93	-2.45	-3.03
miR-146b-3p	2.61	2.51	0.031	0.036	0.93	2.41	2.42
miR-302c	1.44	1.39	0.036	0.035	0.93	2.33	2.44
miR-935	1.64	1.77	0.043	0.043	0.93	2.24	2.31
miR-134	-1.42	-1.41	0.048	0.038	0.93	-2.18	-2.39
miR-127-3p	-1.45	-1.46	0.049	0.041	0.93	-2.16	-2.35

* Summary of miRNAs that overlap in both analyses, LIMMA and ANOVA; the level of significance was determined as $p < 0.01$, the trend for significance was determined as $0.01 < p < 0.05$;

[1] **FC** = Fold change = Expression E/Expression C. Negative FC means down-regulated miRNAs in; endometriosis vs. controls, positive FC – up-regulated miRNAs;

[2] **p-value** – probability of significant difference between the groups, not corrected for multiple testing;

[3] **adj. p-value** – associated p-value after adjustment for multiple testing by Benjamini and Hochberg's method;

[4] **t** = t-statistics;

miRNAs significant by both methods ($p < 0.01$) are presented in bold.

Table 6-6 Dysregulated miRNAs in eutopic endometrium in asymptomatic (n = 4) vs. symptomatic controls (n = 4)*

ID	FC[1]	AveExpr[2]	t[3]	p-value[4]	adj.p-value[5]	B[6]
miR-412	-2.41	3.62	-4.62	0.000	0.39	-3.35
miR-1256	1.92	2.23	3.52	0.004	0.95	-3.68
miR-934	-2.17	3.10	-3.44	0.004	0.95	-3.71
miR-148a*	-2.55	3.76	-3.11	0.008	0.95	-3.83
miR-106a*	2.09	2.50	3.01	0.010	0.95	-3.87
miR-671-3p	-2.52	5.22	-2.85	0.014	0.95	-3.93
miR-29a*	-1.59	2.05	-2.83	0.014	0.95	-3.94
let-7g*	-2.87	4.92	-2.77	0.016	0.95	-3.96
miR-454*	1.62	2.46	2.74	0.017	0.95	-3.97
miR-18b*	-1.65	3.20	-2.71	0.018	0.95	-3.99
miR-1322	1.49	2.27	2.67	0.019	0.95	-4.00
miR-1305	-1.55	2.88	-2.66	0.019	0.95	-4.00
miR-1294	-1.87	3.07	-2.61	0.021	0.95	-4.02
miR-548f	1.89	2.87	2.60	0.022	0.95	-4.03
miR-429	-3.89	4.55	-2.59	0.022	0.95	-4.03
let-7f-1*	2.45	4.13	2.57	0.023	0.95	-4.04
miR-219-2-3p	-1.77	2.71	-2.53	0.025	0.95	-4.05
miR-374a	-2.56	3.43	-2.48	0.027	0.95	-4.07
miR-519b-3p	-1.51	4.21	-2.44	0.029	0.95	-4.09
miR-421	-1.91	7.86	-2.43	0.030	0.95	-4.09
miR-140-3p	1.61	11.93	2.42	0.031	0.95	-4.10
miR-217	1.57	2.46	2.40	0.032	0.95	-4.11
miR-1296	1.59	7.32	2.30	0.038	0.95	-4.15
miR-181d	-1.74	8.23	-2.29	0.039	0.95	-4.15
miR-212	-1.57	7.22	-2.25	0.042	0.95	-4.17
miR-624*	1.43	2.38	2.23	0.043	0.95	-4.18
miR-7	-1.61	2.43	-2.23	0.044	0.95	-4.18
miR-567	1.50	2.76	2.23	0.044	0.95	-4.18
miR-200c*	-2.02	5.87	-2.22	0.044	0.95	-4.18
miR-367*	1.44	2.33	2.17	0.049	0.95	-4.20
miR-301a	-3.02	4.13	-2.17	0.049	0.95	-4.20

* Summary of miRNAs that overlap in both analyses, LIMMA and ANOVA; the level of significance was determined as $p < 0.01$, the trend for significance was determined as $0.01 < p < 0.05$;

[1] **FC** = Fold change = Expression E/Expression C. Negative FC means down-regulated miRNAs in endometriosis vs. controls, positive FC – up-regulated miRNAs;

[2] **AveExpr** - average normalised intensity values for each probe over all the arrays;

[3] **p-value** – probability of significant difference between the groups, not corrected for multiple testing;

[4] **adj. p-value** – associated p-value after adjustment for multiple testing by Benjamini and Hochberg's method;

[5] **t** = t-statistics;

[6] **B** –log-odds that the target is differentially expressed (B statistics).

6.4 Discussion

Endometrium is a complex, dynamic, steroid hormone-responsive tissue that undergoes highly organized cyclic structural changes in preparation for embryo implantation during the reproductive years. It has been suggested that miRNAs regulate endometrial function in a steroid-dependent manner and mediate cross talk between the uterine epithelium and stroma.

The eutopic endometrium of women with endometriosis shares changes with ectopic tissue and these changes are not found in the eutopic endometrium of disease-free women, suggesting that the primary defect in endometriosis are to be found in the eutopic endometrium (530). MiRNAs have been profiled and compared from eutopic endometrium from women in the secretory phase of the menstrual cycle of women with and without endometriosis (206). This study revealed down-regulation of miR-9 and miR-34 miRNA families in endometriosis, reported to target BCL2 gene encoding for anti-apoptotic protein and p53 gene, encoding for oncoprotein TP53, respectively. Further, comparison of eutopic endometrium from women with either mild or severe disease revealed an increased expression of miR-21 and DICER in severe disease (531).

In the present study, array based miRNA global profiling revealed differentially expressed miRNAs in secretory eutopic endometrium in women with endometriosis. We profiled miRNAs in whole endometrium, including both endometrial and stromal compartments, in well characterised prospectively recruited subsets with surgical confirmation of having endometriosis or being disease-free. This experiment was conducted independently of the circulating miRNAs profiling. Biopsy specimens were obtained during secretory phase of the menstrual cycle to assure sampling of sufficient amount of endometrial lining which is considerably thicker at this stage of the cycle. Also, we aimed to enable comparison with the previously published work on endometrial miRNA profiling which was performed on secretory endometrium (206).

Only a small number of miRNAs appeared to be differentially expressed in eutopic endometrium in women with endometriosis in our study. However, our results revealed no correlation with the previously published data (206). This could be explained by differences in samples processing, in detection platforms as well as by differences in the type of control subsets. Disease-free controls in the study conducted by Burney et al., included endometrial

tissue samples obtained from women undergoing hysterectomy for uterine leiomyomas and retrieved from the tissue bank, whereas in our study controls were prospectively enrolled women without uterine pathology. Although none of the leiomyoma-associated miRNAs (528) appeared to be differentially expressed in this study, allowing the authors to assume that leiomyomas were unlikely to affect expression of endometrial miRNAs, other characteristics such as acute or chronic co-morbidities could be an issue. Moreover, the previous study included only moderate-severe forms of endometriosis, whereas endometriosis in our study was mainly of minimal-mild form (4 out of 5 patients). There are recent reports of molecular differences in the endometrium from women with mild vs. severe endometriosis (531). These differences could reflect the miRNA signatures observed in both studies.

The results of this study showed little concordance with the previously published studies that compared miRNA expression between eutopic and ectopic endometrium in endometriosis (207-208) and unpaired eutopic – ovarian endometrioma samples (205). These studies compared 2 different tissue types in women with endometriosis and whereas all of our experiments have compared the same tissue types between women with and without endometriosis, thus, miRNA profile would be expected to be different. Inherent differences in the sample population and in patient selection criteria as well as technical differences in these studies could also contribute to these divergent findings. Together this emphasises the need for further work in the miRNA field involving larger numbers of women.

Our results did not reveal a correlation between eutopic endometrium and the plasma miRNA profile in endometriosis, suggesting that miRNAs in plasma do not reflect a passive release from eutopic endometrial tissue. Growing evidence suggests that circulating miRNAs are actively secreted by cells within the micro vesicles, micro particles, and protein complexes via complex and still largely undisclosed mechanism (200, 214-219). Nearly 30% of the released miRNAs *in vitro* and *in vivo* do not reflect the expression profile found in donor cells (221). It is thus possible that different stimuli alter the number, composition and origin of microparticles (222), and modify the release of miRNAs (223), supporting the assumption that packaging and extracellular transport of miRNAs is highly regulated process.

Changes in eutopic endometrium may be secondary to the peritoneal and plausibly peripheral circulation alterations associated with endometriosis. Exosomal miRNAs have been demonstrated to promote gene silencing in a similar fashion to cellular miRNAs (220, 226). Furthermore, microvesicles isolated from the circulation of atherosclerotic patients, were

noted to induce response in endothelial cells similar to those observed in cells from atherosclerotic lesions, suggesting involvement of circulating miRNAs in mediation of crosstalk between the donor and target cells (223). Thus, circulating miRNAs may hypothetically serve as messengers to influence gene transcription and modulate cell function of eutopic endometrium through receptor-ligand interactions. In this situation, endometrial cells would not be expected to have a profile similar to plasma. In animal models eutopic endometrium changes were observed after induction of endometriosis (53, 62, 68-69), suggesting that endometrial alterations are likely related to the disease progression, rather than an initiating pathogenetic event.

Interestingly, hierarchical clustering of differentially expressed miRNAs in secretory endometrium samples from our study showed segregation not only by endometriosis status, but also by the type of control, i.e. the miRNA profile was different when disease-free women with pelvic pain were compared to those without pain. One of the possible explanations for this finding is that the subgroup of endometriosis-free controls with pelvic pain could have had endometriosis that was overlooked at surgery. Although laparoscopy is still the gold standard, it has some limitations in terms of false negative findings, such as mistaking lesions for corpus luteum cyst, or missing a peritoneal lesion in difficult locations. The positive predictive value of laparoscopic visualization of endometriosis is estimated at about 43-45% and depends on lesion type and location of lesion (119-120), with overall sensitivity of 94% and specificity of 79% of a laparoscopic diagnosis. Moreover, early stages of endometriosis have been described as dynamic event that occurs intermittently in most women, suggesting that the lesions that are not observed in certain women can appear later and vice versa (532).

Another reason may be that chronic pain alters the eutopic endometrium irrespective of endometriosis. Previously, eutopic endometrium and myometrium in women with endometriosis has been characterised by increased density of small unmyelinated sensory C nerve fibres (533). These nerve fibres were also found in ectopic peritoneal endometriotic lesions and in deep infiltrating endometriosis (534) and expressed a wide range of neural function markers, such as VIP, neuropeptide Y, Substance P and calcitonin gene-related peptide, considered responsible for one of the mechanisms of pelvic pain in endometriosis (535). Although the presence of the nerve fibres in functional layer of eutopic endometrium was described as striking characteristic of endometriosis, 6 out of 35 women who did not have endometriosis at laparoscopy, displayed endometrial nerve fibres which was strongly

associated with severe pain and infertility (159). It is possible that the neural markers and associated active substances are increased in women with pelvic pain and modulate miRNA profile.

In summary, the distinctive miRNA profile in endometrium revealed a set of miRNAs unique for endometriosis. Although different miRNAs were differentially expressed when comparisons with different type of controls were applied, over-expression of miR-551a appeared to be consistently unique to endometriosis. MiR-551a is a member of the cancer inhibitory miRNA family and its down-regulation has been found in prostate cancer (536), in childhood acute lymphoblastic leukaemia (537), and in gastric cancer (538). Although the role of this miRNA in endometrium and in endometriosis is still unclear, the targets of miR-551a were found to be involved in the molecular events associated with the disease. The gain of function *in vitro* experiment with mimics of miR-551a, demonstrated the suppressive effect of miR-551a on phosphatase regenerating liver-3 (PRL-3) gene, which is associated with metastasis in gastric cancer, and is believed to play a causative role by promoting tumour cell motility, invasion, and metastasis (538). PRL-3 is over-expressed in endometriotic tissue compared to eutopic endometrium in endometriosis or in healthy controls and was associated with the progression and recurrence of endometriosis (539). It is possible that the elevated levels of miR-551a found by us in eutopic endometrium in endometriosis represent a compensatory mechanism to suppress PRL-3 gene in endometrium. As indicated by data obtained from miRBase, the predicted targets of miR-551a include transcripts that have a functional relevance in endometriosis. For example, one of the targets is Latrophilin 1, which encodes for a subfamily of G-protein coupled receptors that function in both cell adhesion and signal transduction. Latrophilin 1 has been identified down-regulated in eutopic endometrium in endometriosis compared to ectopic endometriotic lesions (83), which is in agreement to up-regulated miR-551a in our study. Another target, zinc finger protein 36 (ZFP36), is involved in regulation of apoptosis and modulates TNF-α-induced regulation of IL-6 and IL-8, both of which were found to be dysregulated in eutopic endometrium in endometriosis (540-541). Members of ZFP36 family, particularly ZFP36l1 were demonstrated to inhibit VEGF translation in mice and thus down-regulation of this transcript is required for angiogenesis (542), which is one of the well-described events in pathogenesis of endometriosis (543). Increased levels of VEGF were detected in eutopic endometrium (544), in peritoneal fluid (545) and blood of women with endometriosis (546). Finally, v-erb-erythroblastic leukaemia viral oncogene

homolog 4 (ERBB4) which is targeted by miR-551a, is a member of the Tyr protein kinase family and EGFR or subfamily that encodes for membrane protein able to induce a variety of cellular responses including mitogenesis and differentiation (547), which are likely to play a role in endometriosis.

As well as providing a better understanding of molecular events involved in endometriosis, identification of the miRNA profile in eutopic endometrium could provide a basis for a diagnostic testing via a minimally invasive endometrial biopsy procedure. The comparison between women with pelvic pain ± infertility with and without endometriosis revealed 2 differentially expressed miRNAs, miR-551a and miR-148a*, which might become promising as endometrial biomarkers of the disease and require further investigation.

6.5 Limitations

The main limitations of the current study include the small numbers in each group and lack of verification by singleplex PCR. Moreover, the current study is descriptive and does not examine the effect of miRNAs in endometrium. Further functional studies are required to determine the role of miRNAs in eutopic endometrium in endometriosis.

None of the miRNAs identified in the current study passed correction for multiple testing, which is most likely explained by the limited power of the study. To minimise the risk of false positive results, we applied the stringent criteria including higher threshold for the level of significance ($p < 0.01$) and selection of the results at the intersection of 2 types of statistical analyses. Having said that, we cannot confidently state that the observed differences are free of bias and do not contain expression differences found by chance.

6.6 Conclusions

In summary, this pilot study is the first report of the large-scale miRNA profile in secretory eutopic endometrium in association with endometriosis that addresses dysregulation patterns of miRNAs in comparison to laparoscopically proven endometriosis-free population. The results of the current study indicate that altered miRNA expression profile in eutopic endometrium provides putative candidates for further investigation of pathogenetic effects associated with the disease as well as for the targeted drug discovery for endometriosis-based effects on endometrium. Our results also provide an initial insight into discovery of miRNA-based diagnostic test for endometriosis, which can incorporate endometrial biopsy either solely or in combination with the blood test.

Chapter 7
Plasma miRNAs as a non-invasive diagnostic tool for endometriosis

7.1 Introduction

The current diagnosis of endometriosis relies heavily on visualisation of the endometriotic lesions at surgery. This is a procedure which carries considerable costs, confers risks such as bleeding, infection and damage to adjacent organs and is one of the reasons for a significant diagnostic delay (97, 99, 101, 548). Increased time between the onset of symptoms and a diagnosis of endometriosis has been associated with: 1. worsened prognosis for fertility, 2. increased physical and psychological morbidity due to a long duration of undiagnosed pain, and 3. increased risk of repeated operative procedures (102). The younger a patient is when symptoms start, the longer the delay to diagnosis of endometriosis (100). The development of a non-invasive diagnostic test for endometriosis is likely to reduce the long delay between onset of symptoms and diagnosis and to improve patient quality of life. An effective non-invasive test for endometriosis would also promote clinical research, allowing development and testing of novel therapeutic approaches. Despite the numerous studies that have been performed in an attempt to identify a clinically useful marker for endometriosis that can substitute for current invasive test for endometriosis, none are sensitive and specific enough to be implemented routinely in clinical practice (133, 153).

The current study explores the potential usefulness of circulating miRNAs as a non-invasive diagnostic test for endometriosis. We developed methods to accurately and reproducibly measure miRNA levels in plasma (Chapter 3) and identified a distinctive miRNA profile in plasma of women with endometriosis by using miRNA arrays (Chapter 4). We also demonstrated that the expression of the endometriosis-modulated miRNAs does not vary with the stage of the menstrual cycle (Chapter 4).

Here we present a prospective cohort diagnostic study in which we apply quantitative qRT-PCR measurements to a panel of endometriosis-associated miRNAs isolated from plasma of well characterised women with and without endometriosis. A panel of miRNAs that most strongly differentiate women with and without endometriosis was selected for further validation. The resulting miRNA profiles in plasma samples are correlated with diagnosis, demographic and clinical parameters of the study population.

7.2 Materials and Methods

7.2.1 Study design

This study was divided into 3 phases: phase I – marker discovery, phase II – marker selection and phase III – large-scale validation and optimisation of the diagnostic model.

7.2.1.1 Phase I - Marker discovery

The marker discovery phase was a case-control study based on global miRNA profiling in plasma of women with endometriosis and disease-free controls. Importantly, we used the experimental array data presented in Chapter 4, but the approach to selection of the putative targets and the group analyses was different, as described in Chapter 3. Differentially expressed miRNAs identified at this stage were used for further analyses.

7.2.1.2 Phases II-III – Marker selection and large-scale validation

This was a study of prospective cohort design and included consecutive patients undergoing laparoscopy for diagnosis and treatment of pelvic pain or infertility. In this set of experiments, the putative miRNA markers identified in phase I were verified first in a small sample set (phase II) and further validated and optimized on a larger set of samples (phase III).

7.2.2 Study participants

Two different sets of patients were recruited for the study: symptomatic patients with surgically verified endometriosis as well as asymptomatic healthy controls (for the study phase I) and a cohort of symptomatic women with surgical diagnosis of either endometriosis or disease-free status (for the study phases II and III). With the exception of the asymptomatic healthy controls, the participants included in both sets were recruited prior to the surgical diagnosis.

7.2.2.1 Phase I – marker discovery

The endometriosis group included 8 women with symptoms suggestive of endometriosis (pelvic pain ± infertility) with surgical confirmation of the diagnosis and 8 asymptomatic disease-free women. The patients' characteristics as well as the inclusion and exclusion criteria are presented in Chapter 4.

7.2.2.2 Phases II-III – Marker selection and large-scale validation

A cohort of additional women with clinically suspected endometriosis who were admitted for laparoscopy for investigation of pelvic pain ±infertility was prospectively enrolled. The patients were identified at a preadmission outpatient visit and were operated between March 2010 and November 2011.

The inclusion criteria were: reproductive age (16-48), clinical suspicion of endometriosis, and willingness to undergo surgery for investigation and management of chronic pelvic pain. Women on hormonal contraception or hormonal treatment were accepted. The surgery was carried out at any stage of the menstrual cycle. Patients were excluded if their age was outside of the inclusion criteria, they had a post or current malignant condition or history of chemo- or radiotherapy, had signs of ongoing acute infectious disease, or refused to participate in the study.

A detailed medical history was taken from all participants. This included menstrual history, pain symptoms, infertility, number and outcome of previous pregnancies, chronic diseases, current medications, previous surgery and family history. Blood samples from all participants were collected on the day of surgery prior to anaesthesia. The diagnosis of endometriosis was made at surgery by direct visualization of the pelvic cavity. Suspected lesions were sent for histopathology. The severity of endometriosis was classified according to the rASRM classification system. Every surgical team included a senior gynaecologic laparoscopist with more than 10 years' experience in the diagnosis and management of endometriosis.

7.2.3 Processing of the samples and miRNA profiling

Blood processing, miRNA extraction and quality assessment of the samples was performed using the techniques described in Chapter 3. TLDA A and B were used for a screening of miRNA expression in phase I of the study. Only miRNAs that significantly differed between the groups and passed a test for multiple test correction were considered for the subsequent validation step by the singleplex assays with qRT-PCR in phases II and III. All samples were processed under similar conditions and run in single replicate for the array experiment and in triplicate measurements for qRT–PCR for validation of selected putative target miRNAs. The techniques of the miRNA profiling and approach to the data analysis are presented in Chapter 3.

7.2.4 Selection of targets to explore for a diagnostic test

7.2.4.1 Study phase II

Firstly, miRNAs identified in the array experiment were assessed using singleplex qRT-PCR in a small randomly selected set of plasma samples from 10 women with endometriosis and 10 controls. At this stage of analysis, we considered any changes that occurred between disease-free and endometriosis groups or between disease-free and any of the subgroups of endometriosis stratified by the severity of the disease to be of interest. Differences were considered of interest if they appeared either at the level of statistical significance or showed a trend for significance, which was defined as $0.05 < p < 0.1$. A type I error of 10% was considered acceptable for selection of miRNAs chosen for further follow up, given the explorative nature of this experiment, with the aim of proceeding to a further validation step.

7.2.4.2 Study phase III

Markers that appeared to be differentially expressed at the level of statistical significance or by showing a trend for significance were validated in a large-scale set of plasma from women within the same cohort (49 women with endometriosis and 19 controls).

7.3 Results

7.3.1 Phase I - Marker discovery

A description of the participants recruited for this experiment is presented in the results section of Chapter 4; the characteristics of the participants are summarised in Table 4-1.

In total, out of 674 screened miRNAs, 325 were detectable in the blood in > 70% of the samples and were used for the subsequent analyses (221 miRNAs on card A and 104 miRNAs on card B). The performance of the arrays and quality control of the samples are presented in Chapter 4.

We found a total of 76 miRNAs that were differentially expressed in plasma in women with and without endometriosis. Of these, 25 miRNAs remained significant after correction for multiple testing (13 miRNAs on card A and 12 miRNAs on card B) and were selected for further validation by qRT-PCR (Table 7-1). Amongst these miRNAs, 16 were up-regulated and 9 were down-regulated in endometriosis. None of the selected 25 endometriosis modulated miRNAs showed fluctuation in the expression across the menstrual cycle in the endometriosis or in the control group. The panel included RNU-6B, a small nuclear non coding RNA also known as U6.

Even though it is not formally classified as miRNA, we included RNU-6B in validation experiments as a potential marker.

Table 7-1 Dysregulated miRNAs in plasma in endometriosis, study phase I

miR name	logFC[1]	AveExpr[2]	t[3]	p-value[3]	adj. p-val[4]	B[5]
miR 574-3p	4.82	23.07	5.94	< 0.001	< 0.001	6.55
miR 155	-5.50	26.30	-5.87	< 0.001	< 0.001	6.31
miR 23a	1.95	28.30	5.72	< 0.001	< 0.001	5.82
miR 128	-1.23	25.13	-4.43	< 0.001	0.004	1.60
miR 362-3p	-1.00	29.09	-4.23	< 0.001	0.006	0.98
miR 148a	-0.81	24.07	-4.16	< 0.001	0.006	0.78
miR 148b	-0.59	25.02	-3.98	< 0.001	0.009	0.22
miR 30c	-0.59	18.27	-3.56	< 0.001	0.026	-1.01
miR 502-3p	-0.86	28.65	-3.55	< 0.001	0.026	-1.01
miR 433	1.02	25.83	3.50	0.001	0.028	-1.16
miR 425	0.58	22.00	3.38	0.001	0.036	-1.48
miR 135b	-0.81	30.75	-3.35	0.002	0.037	-1.59
miR 133a	-1.00	23.63	-3.24	0.002	0.046	-1.87
miR-662	-3.11	30.50	-6.99	< 0.001	< 0.001	9.20
miR-93*	-1.50	23.27	-6.09	< 0.001	< 0.001	6.45
miR-29a*	1.47	31.52	4.58	< 0.001	0.002	1.81
RNU6B	1.21	31.66	4.14	< 0.001	0.007	0.53
miR-378*	-0.96	30.68	-3.41	0.002	0.039	-1.50
miR-923	1.95	23.19	3.39	0.002	0.039	-1.54
miR-630	1.91	31.35	3.32	0.002	0.040	-1.73
miR-625*	-0.93	21.47	-3.23	0.003	0.040	-1.95
miR-661	2.00	28.30	3.20	0.003	0.0340	-2.02
miR-550	-0.87	30.68	-3.12	0.003	0.044	-2.24
miR-10b*	-1.42	28.06	-3.19	0.003	0.040	-2.04
miR-145*	-0.95	29.23	-3.07	0.004	0.044	-2.35

[1] **log FC** - log2(-fold change); FC = 2^{-logFC};

[2] **AveExpr** - average normalised Cq values across all arrays in the experiment;

[3] **t** - moderated t-statistics;

[4] **adj. p-value** –associated p-value after adjustment for multiple testing by Benjamini and Hochberg's method;

[5] **B** - B-statistics = log-odds that the target is differentially expressed.

7.3.2 Phases II-III – Marker selection and large scale validation

7.3.2.1 Study participants

Out of 76 women with clinically suspected endometriosis that were recruited for the study, 8 participants were excluded, because the blood samples collected from these women underwent haemolysis and were inappropriate for testing. In total, samples from 68 women were included for the analysis: 42 women were recruited from a private practice and 26 women from a public system. Altogether, 49 women were diagnosed with endometriosis, of them 34 women with minimal-mild disease (18 women at stage I, 16 women at stage II) and 15 women with moderate-severe disease (12 women at stage III, 3 women at stage IV), according to the rASRM endometriosis classification. Pelvic biopsy was performed in 46 (67.5%) women and confirmed the diagnosis based on visual inspection.

The mean age of the study population was 31.4 ± 9.4, ranging 16 - 48 years. The mean BMI of the study population was 25.4 ± 5.0, ranging 17.0 - 41 kg/m^2. The baseline characteristics of this population are summarised in Table 7-2. No significant differences in age, BMI, smoking, stage or pattern of the menstrual cycle, general medical history, presence of polycystic ovary syndrome (PCOS) or family history of endometriosis were found when the women with and without endometriosis were compared. Few women used hormonal medications (predominantly the combined oral contraceptive pills in our study population), however hormonal use was significantly more common in women diagnosed with endometriosis than in disease-free women.

The Spearman correlation coefficients between expression levels of each of the tested miRNAs and hormonal treatment were below 0.2 in both endometriosis and in control groups, which indicated no association between hormonal treatment and miRNA expression and thus suggested that hormonal treatment is not a confounding variable in this context.

The endometriosis group had a larger proportion of nulliparous women compared to the control group, however a substantial proportion of women had never tried to conceive [26 (56.5%) in endometriosis group and 4 (22.2%) in the disease-free group]. An analysis of women who attempted conception showed similar parity and miscarriage rates for both groups.

Table 7-2 General characteristics of the study population

Characteristics	Endometriosis, n = 49	Controls, n = 19	p-value
Age, years			
Mean ± SD	30.04 ± 8.95	34.74 ± 9.84	0.069*
Median [range]	31 [16-45]	36 [22-48]	
BMI, kg/m^2			
Mean ± SD	25.13 ± 4.61	25.98 ± 6.05	0.617*
Median [range]	24 [18.1-37.1]	24.4 [17-40.6]	
Smokers, n (%)	5 (10.9%)[2]	4 (25%)[3]	0.219***
Stage of menstrual cycle, n (%)			
Early proliferative (day 1-8)	12 (24.5%)	4 (21.05%)	0.317***
Late proliferative (day 9-14)	12 (24.5%)	2 (10.5%)	
Luteal (day 15-28)	12 (24.5%)	9 (47.4%)	
Undetermined	13 (26.5%)	4 (21.05%)	
Pattern of menstrual cycle, n (%)			
Regular (21-35 days)	41 (85.4%)[1]	16 (84.2%)	0.360***
Irregular	7 (14.6%)	2 (10.5%)	
Amenorrhea	0 (0%)	1 (5.3%)	
Medical history, n (%)			
No chronic diseases	40 (81.6%)	13 (68.4%)	
Asthma	3 (6.25%)	1 (5.3%)	
Hypothyroidism	2 (4.1%)	0 (0%)	
Diabetes Mellitus	0 (0%)	1 (5.3%)	0.327***
Rheumatoid arthritis	0 (0%)	1 (5.3%)	
Anxiety disorder	3 (6.1%)	2 (10.5%)	
Heart transplant	1 (2%)	0 (0%)	
IBS	0 (0%)	1 (5.3%)	
PCOS, n (%)	5 (10.2%)	1 (5.3%)	1.0***
Family history of endometriosis, n (%)			
No endometriosis	33 (71.7%)[2]	13 (76.5%)[4]	1.0***
Endometriosis	13 (28.2%)	4 (23.5%)	
Hormonal medications, n(%)	19 (39.6%)	2 (10.5%)	0.022***
Parity, n (%)			
0	33 (70.2%)[5]	7 (38.9%)[6]	0.036***
1	7 (14.9%)	7 (38.9%)	
2	6 (12.8%)	2 (11.1%)	
3	1 (2.1%)	2 (11.1%)	
Parity[7], n (%)			
0	7 (33.3%)	3 (21.4%)	
1	7 (33.3%)	7 (50%)	0.451***
2	6 (28.6%)	2 (14.3%)	
3	1 (4.8%)	2 (14.3%)	
Miscarriages[7], n (%)			
0	13 (65%)	6 (42.9%)	
1	6 (30%)	4 (28.6%)	0.279***
2	0 (0%)	1 (7.1%)	
3	0 (0%)	2 (14.3%)	
4	1 (5%)	1 (7.1%)	

*Mann Whitney U test; *** Fisher's exact test;

[1] not recorded for 1 patient (2%);

[2] not recorded for 3 patients (6%);

[3] not recorded for 3 controls (16%);

[4] not recorded for 2 controls (11%);

[5] not recorded for 2 patients (4%);

[6] not recorded for 1 control (5%);

[7] in women who attempted conception, n = 34.

The clinical parameters, which included symptoms, duration of symptoms, and type of infertility, are presented in Table 7-3. All the women included in the study suffered from chronic abdominal pain. The majority of women in both groups experienced pain from the age of 12-16 years (30 (63.8%) in endometriosis group and 9 (52.9%) in disease-free women) with no difference between the duration of the pain symptoms between the groups.

An assessment within the cohort of women who had tried to conceive, demonstrated no difference in infertility rate or infertility type (primary vs. secondary infertility) between the 2 groups. Women with endometriosis and disease-free women had comparable rates of abnormal uterine bleeding (heavy menstrual or intermenstrual bleeding) and headaches.

Table 7-3 Clinical characteristics of the study population

Characteristics	Endometriosis, n = 49	Controls, n = 19	p-value
Clinical setting, n (%)			< 0.001***
Private practice, n = 42	37 (75.5%)	5 (26.3%)	
Public system, n = 26	12 (24.5%)	14 (73.7%)	
Symptoms, n (%)			
Pain	49 (100%)	18 (100%)	
Infertility	10 (21.7%)[1]	9(50%)2	0.029***
Infertility[3]	10 (50%)	9 (64.3%)	0.495***
Abnormal uterine bleeding	10 (20.8%)[4]	6 (33.3%)[2]	0.340***
Duration of pain symptoms			
Since teenage years, n (%)	30 (63.8%)[5]	9 (52.9%)[6]	0.563***
Duration, years			
Mean ± SD	9.06 ± 6.96	11.18 ± 7.9	0.305**
Median [range]	7[1-25]	10[3-28]	0.240*
Types of infertility[7], n (%)			
Primary	3 (30%)	0 (0%)	0.224***
Secondary	6 (60%)	6 (66.7%)	
Recurrent miscarriages	1 (10%)	3 (33.3%)	
Migraines, n (%)	20 (45.5%)[8]	10 (55.6%)[2]	0.579***
Previous laparoscopies, n (%)			0.023***
Endometriosis found	14 (29.2%)[4]	4 (22.2%)[2]	
Endometriosis not found	3 (6.3%)	6 (33.3%)	
Type of surgery, n (%)			0.206***
Laparoscopy	45 (91.8%)	15 (78.9%)	
Laparoscopic hysterectomy	4 (8.2%)	4 (21.1%)	
Other findings at surgery			
Fibroids	3 (6.1%)	2 (10.5%)	0.541***
Endometrioma	8 (16.3%)		
Hydrosalpinx	1 (2%)	1 (5.3%)	0.632***
CIN III	1 (2%)	0 (0%)	

*Mann Whitney U test; ** Student t-test; *** Fisher's exact test; [1] not recorded for 3 patients (6%);
[2] not recorded for 1 control (5%); 3 in women who attempted conception, n = 34;
[4] not recorded for 1 patient (2%); [5] not recorded for 2 patients (4%); [6] not recorded for 2 controls (11%);
[7] within infertile population, n = 19;
[8] not recorded for 5 patients (10%).

A total of 27 women had previous laparoscopic surgery for investigation of pelvic pain. Women in the endometriosis group had a higher rate of previous laparoscopies with positive findings of endometriosis and a lower rate of laparoscopies negative for endometriosis compared to controls p = 0.023, Fisher's exact test. In 18 women a diagnosis of endometriosis was confirmed at previous surgery (14 (29.2%) from endometriosis and 4 (22.25) from disease-free group) and in 9 women the endometriotic lesions were not detected at previous surgery (3 (6.3% in endometriosis and 6 (33.3%) in the disease-free group).

There was no statistically significant difference in type of surgery, laparoscopy or laparotomy, between the groups. There was a comparable rate of fibroids and hydrosalpinges between the 2 groups of women. Endometrioma was present in 8 women (16.3%) in the endometriosis group.

There were no statistically significant differences between women attending private practice or the public health system for any of the considered demographic, clinical or surgical variables. Exception was the rate of infertility and miscarriages that were more prevalent in patients attending public service (data not shown). Patients attending the private practice had a higher rate of diagnosis, especially for severe disease, however this is unsurprising as this practice predominantly received tertiary referrals.

7.3.2.2 Quality control of the samples and miRNA assays

The average Cq values of the spiked-in synthetic cel-miR-54 in all selected samples were 19.5 ± 0.6, 20.9 ± 0.5, 20.2 ± 1.2, 20.7±0.4 (mean ± SD, presented separately per each PCR reaction plate), with a coefficient of variance (CV) of 3%, 2.5%, 5.9% and 1.9%, respectively. The above results suggested a satisfactory and comparable RNA extraction efficiency across the samples. The miRNA across the plasma samples accounted for 55%-92% of total small RNAs with median concentration of 346.3 pg/µl, ranging 154-796 pg/µl. The summary of the samples with cel-miR-54 levels and Bioanalyzer results are presented in the Supplemental Material on CD, Table 5S.

The SD of the sample replicates for each of the assays for investigated miRNAs did not exceed 0.5 Cq. The amplification efficiency for the majority of included assays was between 90% and 110%, ranging from 88% to 175%. A Spearman's Rank Order correlation was run in 20 samples for each detector to determine the relationship between corrections to efficiency of amplification vs. assumption of efficiency of 100%. There was a strong, positive correlation

between corrected and uncorrected raw CT values for each detector, which was statistically significant at the 0.01 level, $r_s = 1$ for each miRNA, implying that a correction for efficiency did not change the expression pattern of tested miRNAs.

7.3.2.3 Selection and validation of the endogenous controls for quantification of plasma miRNAs

For the purpose of this study, we used a systematic approach to identify suitable endogenous controls, as described in Chapter 3. First, we identified a set of putative normalisers via application of GeNorm algorithm in the array experiment. The expression stability M value was lowest for 3 miRNAs for card A out of the 43 candidates and for 3 miRNAs for card B out of the 24 candidates. According to the pair-wise variation V value, 2 endogenous controls turned out to be sufficient to normalize expression levels for each of the array cards (V 2/3 = 0.012 for card A and V 2/3 = 0.008 for card B). Interestingly, mammalian U6, used as a stable and reliable endogenous control in other studies (211, 549), had the highest M-value and was less abundant compared to the other miRNAs identified by GeNorm. Two endogenous controls from card A (miR-28-5p and miR-30b) and 3 endogenous controls from card B (miR-30a, miR-30d and miR-30e-3p) were selected for validation and were subjected to qRT-PCR assay using 20 samples. The assessment of validity of the endogenous controls included the following: 1 – each of the selected candidate controls demonstrated negative NTC signals; 2 - the serial dilutions of the purified RNA samples revealed excellent assay linearity; 3 - each of the selected candidate endogenous controls was readily detected across the samples ; 4 –all the candidate controls had small variation across the samples with coefficient of variance < 0.05; 5 – None of the putative endogenous controls exhibited expression differences between the groups (endometriosis vs. controls). Notably, one of the putative endogenous controls miR-30c was also identified to be one of the significantly differentially expressed miRNA when quantile normalisation was performed. Considering that miR-30c did not exhibit any superior qualities to other normalisers, we decided to treat it as a target and omitted it from the list of the normalisers. Given that using more than one endogenous control ensures the least variation due to untested samples and conditions (305), we selected miR-28-5p and miR-30b as normalisers for the assays originated from card A and miR-30a, miR-30d and miR-30e-3p for normalising the assays form card B. A geometric mean of these miRNAs was selected as a normalisation control for quantification of plasma miRNAs in single qRT-PCR experiments.

7.3.2.4 Marker selection

To validate the putative markers for endometriosis identified in the array experiment, we first examined the expression levels of the candidate miRNAs by using qRT-PCR on a small set of randomly selected plasma samples from 10 women with endometriosis and 10 disease-free women. The investigated candidate miRNAs included 13 miRNAs identified on card A (miR-128, miR-133a, miR-135b, miR-148a, miR-148b, miR-155, miR-23a, miR-30c, miR-362-3p, miR-425, miR-433, miR-502-3p, miR-574-3p) and 11 miRNAs identified on card B (miR-662, miR-93-3p, miR-29a-5p, miR-RNU6B, miR-378a-5p, miR-630, miR-625-3p, miR-661, miR-550a-5p, miR-10b-3p, miR-145-3p). MiR-923, identified on the array card B, has been removed from the Sanger database v.18 as it appeared to be a fragment of the 28S ribosomal RNA, and therefore was not included in the validation experiments.

Our data indicated that miR-135b, miR-662, miR-29a-5p and miR-RNU6B were expressed below the established detection threshold in both endometriosis and disease-free groups and thus were excluded from the subsequent analyses. The circulating expression levels of the remaining 20 miRNAs were analysed (12 miRNAs identified on the card A of the array and 8 miRNAs identified on the card B). The expression levels and fold changes of the candidate miRNAs assessed in the study phase II are summarised in Table 7-4.

Although there was no significant difference in the expression levels of any of the investigated miRNAs between endometriosis and disease-free groups, miR-155, miR-574-3p and miR-630 tended to be down-regulated in endometriosis, exhibiting a trend for statistical significance (FC = 1.8, p = 0.075 and FC = 1.7, p = 0.063 and FC = 1.2, p = 0.075, respectively).

In the endometriosis group, 5 women had minimal–mild disease (rASRM stage I-II) and 5 women had moderate–severe disease (rASRM stage III-IV). The analysis revealed that the expression levels of 4 miRNAs were lower in minimal-mild endometriosis than in disease-free women [miR-155 (FC = 2.3, p = 0.04), miR-30c (FC = 1.3, p = 0.075), miR-425 (FC = 1.9, p = 0.099), 574-3p (FC = 1.9, p = 0.099)] (Table 7-5). There was a tendency towards an increase in the expression levels of these miRNAs in the more severe forms of the disease.

Table 7-4 miRNA expression in plasma in endometriosis (n = 10) and in disease-free women (n = 10); validation of the TLDA results by qRT-PCR*

miRNA	MirBase accession number	Disease-free controls n = 10		Endometriosis n = 10		Average Fold Change	p-value
		Mean ± SEM	Median (range)	Mean ± SEM	Median (range)		
miR-128	MIMAT0000424	1.43 ± 0.46	1.05 (0.68-5.53)	0.97 ± 0.11	0.92 (0.61-1.73)	-1.24	0.393
miR-133a	MIMAT0000427	3.34 ± 1.32	2.04 (0.87-14.34)	3.18 ± 0.44	3.42 (0.67-4.91)	1.21	0.156
miR-148a-3p	MIMAT0000243	0.05 ± 0.03	0.03 (0.02-0.28)	0.03 ± 0.004	0.025 (0.02-0.05)	-1.22	0.796
miR-148b-3p	MIMAT0000759	0.06 ± 0.03	0.03 (0.02-0.03)	0.04 ± 0.003	0.04 (0.02-0.05)	-1.14	0.853
miR-155	MIMAT0000646	1.65 ± 0.45	1.18 (0.63-5.52)	0.87 ± 0.14	0.94 (0.26-1.64)	-1.79	0.075
miR-23a-3p	MIMAT0000078	0.01 ± 0.002	0.01 (0.003-0.02)	0.01 ± 0.001	0.01 (0.004- 0.01)	-1.1	0.684
miR-30c-5p	MIMAT0000244	7.72 ± 0.87	7.88 (3.10-13.51)	6.68 ± 0.40	6.53 (4.36-8.69)	-1.11	0.280
miR-362-3p	MIMAT0004683	0.47 ± 0.33	0.2 (0.03-2.46)	0.15 ± 0.04	0.13 (0.003- 0.04)	-1.46	0.669
miR-425-5p	MIMAT0003393	9.76 ± 4.96	5.84 (1.57-54.05)	3.62 ± 0.58	3.35 (1.80-8.12)	-1.69	0.143
miR-433	MIMAT0001627	0.02 ± 0.01	0.02 (0.003-0.05)	0.02 ± 0.01	0.016 (0.003-0.07)	-1.35	0.606
miR-502-3p	MIMAT0004775	0.04 ± 0.02	0.01 (0.002-0.25)	0.01 ± 0.003	0.01 (0.003-0.04)	-1.38	0.796
miR-574-3p	MIMAT0003239	3.80 ± 0.93	2.690 (1.53-10.74)	2.01 ± 0.24	1.82 (0.88-2.90)	-1.68	0.063
miR-10b-3p (10b*)	MIMAT0004556	0.03E-02 ± 0.58E-03	0.002 (0.001-0.01)	0.15E-02 ± 0.22E-03	0.0015 (0.001-0.002)	-1.21	0.436
miR-145-3p (145*)	MIMAT0004601	4.85E-05 ± 1.48E-05	2.34E-05 (0.9E-05-12.7E-05)	3.91E-05 ± 9.76E-06	3.08E-05 (2.5E-05-8.8E-05)	-1.07	0.631
miR-378a-5p (378*)	MIMAT0000731	2.45E-06 ± 4.87E-07	2.0E-06 (6.8E-07-6.4E-06)	1.93E-06 ± 2.90E-07	2.0E-06 (8.5E-07-3.1E-06)	-1.17	0.562
miR-550a-5p (550)	MIMAT0004800	0.01 ± 0.001	0.01 (0.002-0.01)	0.58E-02 ± 0.11E-02	0.01 (0.002-0.01)	-1.33	0.481
miR-625-3p (625*)	MIMAT0004808	0.23 ± 0.06	0.171 (0.09-0.65)	0.23 ± 0.06	0.19 (0.111-0.521)	1.21	0.280
miR-630	MIMAT0003299	1.82E-06 ± 4.79E-07	0.13E-05 (0.1E-05-0.6E-05)	1.52E-06 ± 4.39E-07	9.76E-07 (7.77E-07-3.95E-06)	-1.20	0.075
miR-661	MIMAT0003324	1.27 ± 0.34	1.0 (0.282-3.370)	1.14 ± 0.45	0.73 (0.23-2.96)	-1.40	0.529
miR-93-3p (93*)	MIMAT0004509	4.75 ± 0.51	4.56 (1.79-7.19)	3.69 ± 0.27	3.84 (2.54-4.51)	-1.12	0.143

* Values are presented as relative expression, mean ± SEM. Fold change represents a relative fold change of endometriosis group compared to disease-free controls. Statistically significant differences between the disease-free controls and endometriosis were determined using Mann-Whitney U test; the level of significance was determined as $p < 0.05$, the trend for statistical significance was determined as $0.05 \leq p \leq 0.099$.

Table 7-5 miRNA expression in plasma set by severity of endometriosis: disease-free controls (n = 10), endometriosis (n = 10), rASRM stage I-II (n = 5), rASRM stage III-IV (n = 5); validation of the TLDA results by qRT-PCR*

miRNA	Mild Endometriosis Mean ± SEM	Fold Change[1]	p-value[1]	Severe Endometriosis Mean ± SEM	Fold Change[2]	p-value[2]
miR-128	0.90 ± 0.11	-1.30	0.371	1.03 ± 0.2	-1.18	0.679
miR-133a	2.51 ± 0.73	-1.12	0.898	3.85 ± 0.35	1.64	0.029
miR-148a	0.03 ± 0.01	-1.31	0.679	0.03 ± 0.01	-1.13	1.0
miR-148b	0.03 ± 0.01	-1.16	1.0	0.04 ± 0.01	-1.13	0.768
miR-155	0.68 ± 0.18	-2.33	0.040	1.05 ± 0.18	-1.37	0.440
miR-23a	0.01 ± 0.001	1.09	1.0	0.01 ± 0.001	-1.32	0.513
miR-30c	5.74 ± 0.38	-1.28	0.075	7.63 ± 0.36	1.05	1.0
miR-362-3p	0.12 ± 0.03	-1.83	0.432	0.19 ± 0.07	-1.16	1.0
miR-425	3.12 ± 0.51	-1.89	0.099	4.12 ± 1.07	-1.51	0.513
miR-433	0.02 ± 0.01	-1.96	0.435	0.02 ± 0.01	1.18	1.0
miR-502-3p	0.01 ± 0.003	-1.53	0.768	0.02 ± 0.01	-1.24	0.953
miR-574-3p	1.84 ± 0.39	-1.88	0.099	2.18 ± 0.30	-1.49	0.206
miR-10b-3p(10b*)	9.3E-04 ± 3.9E-04	-1.36	0.440	0.002 ± 1.6E-04	-1.18	0.679
miR-145-3p(145*)	2.9E-05 ± 2.0E-06	-1.43	0.859	4.5E-05 ± 1.46E-05	1.64	0.310
miR-378a-5p(378*)	1.0E-06 ± 4.0E-07	-1.74	0.273	2.0E-06 ± 3.0E-07	-1.13	1.0
miR-550a-5p(550)	0.01 ± 0.004	-1.09	0.953	0.57E-02 ± 0.92E-03	-1.13	0.310
miR-625-3p(625*)	0.32 ± 0.20	1.53	0.206	0.18 ± 0.03	-1.37	0.679
miR-630	1.0E-06 ± 1.0E-07	-1.22	0.371	2.0E-06 ± 7.0E-07	-1.32	0.055
miR-661	1.08 ± 0.85	-1.46	0.953	1.17 ± 0.63	1.05	0.371
miR-93-3p(93*)	3.72 ± 0.25	-1.09	0.206	3.68 ± 0.41	-1.16	0.310

* Values are presented as relative expression, mean ± SEM. Fold change represents a relative fold change of endometriosis compared to disease-free controls. Statistically significant differences between the disease-free controls and endometriosis were determined using Mann-Whitney U test; the level of significance was determined as $p < 0.05$, the trend for statistical significance was determined as $0.05 \leq p \leq 0.099$;

[1] minimal-mild endometriosis compared to controls;

[2] moderate-severe endometriosis compared to controls.

Severity of disease had a prominent effect on miR-30c, which showed significant up-regulation in moderate-severe endometriosis in comparison to mild-moderate disease (FC = 1.3, p = 0.008). In contrast, miR-630 demonstrated decreased expression with an increased severity of the disease (FC = 1.3, p = 0.055). The expression level of miR-133a did not change in minimal-mild disease but was higher by 1.6 fold in moderate-severe endometriosis than in disease-free subjects (p = 0.029). MiR-145-3p was up-regulated and miR-625-3p was down-regulated in moderate-severe endometriosis, compared to its minimal-mild forms (FC = 1.8, p = 0.056 and FC = 1.6, p = 0.095, respectively).

Thus, in keeping with our criteria for selection of the miRNAs of interest, 8 miRNAs were chosen for further validation: miR-155, miR-574-3p, miR-425, miR-133a, miR-30c, miR-630, miR-145-3p and miR-625-3p, as summarised in Table 7-6.

Table 7-6 Summary of the candidate miRNAs selected in the study phase II for further validation*

Compared groups	miRNA	Fold Change	Fold Change	p-value
Endometriosis vs. Disease-free	miR-155	0.56	-1.79	0.075
	miR-574-3p	0.6	-1.68	0.063
	miR-630	0.83	-1.20	0.075
Minimal-mild Endometriosis vs. Disease-free	miR-155	0.43	-2.33	0.04
	miR-574-3p	0.53	-1.88	0.099
	miR-425	0.53	-1.89	0.099
	miR-30c	0.78	-1.28	0.075
Moderate-severe Endometriosis vs. Disease-free	miR-133a	1.64	1.64	0.029
	miR-630	0.76	-1.32	0.055
Moderate-severe Endometriosis vs. Minimal-mild Endometriosis	miR-30c	1.34	1.34	0.008
	miR-145-3p	1.78	1.78	0.056
	miR-625-3p	0.63	-1.48	0.095

* Fold change represents a relative fold change of endometriosis compared to disease-free controls or between the stages of severity in endometriosis. Statistically significant differences between the disease-free controls and endometriosis were determined using Mann-Whitney U test; the level of significance was determined as $p < 0.05$, the trend for statistical significance was determined as $0.05 \leq p \leq 0.099$.

7.3.2.5 Validation of the selected markers in larger set of the patients

7.3.2.5.1 The expression of the candidate miRNAs in endometriosis

The expression levels of miR-155, miR-574-3p, miR-425, miR-133a, miR-30c, miR-630, miR-145* and miR-625* were measured on a total of 68 plasma samples, including 49 women with endometriosis (18 with rASRM stage I, 16 with rASRM stage II, 12 with rASRM stage III and 3 with rASRM stage IV) and 19 disease-free women (Table 7-7). All the investigated miRNAs were readily amplified and detected in all samples, with the exception of miR-133a, which was undetected in one sample from the disease-free group. Both miR-155 and miR-574-3p were significantly down-regulated in plasma samples from patients with endometriosis compared with those from the disease-free group ($p = 0.009$ and $p = 0.03$, respectively), by 1.44 fold each. Expression of the remainder of the investigated miRNAs did not differ significantly between the endometriosis cohort and disease-free controls.

Table 7-7 Expression of the 8 candidate miRNAs in women with endometriosis (n = 49) and in disease-free controls (n = 19); study phase III*

miRNA MirBase name	MirBase accession number	Expression of miRNAs in Disease-free controls n = 19		Expression of miRNAs in Endometriosis n = 49		Average Fold Change	p-value
		Mean ± SEM	Median (range)	Mean ± SEM	Median (range)		
miR-155	MIMAT0000646	0.10 ± 0.02	0.01 (0.05-0.27)	0.070 ± 0.004	0.07 (0.02-0.13)	-1.44	0.009
miR-574-3p	MIMAT0003239	0.322 ± 0.05	0.23 (0.12-0.78)	0.205 ± 0.013	0.19 (0.08-0.53)	-1.44	0.030
miR-30c-5p	MIMAT0000244	0.62 ± 0.04	0.62 (0.33-0.97)	0.627 ± 0.019	0.63 (0.35-0.97)	1.01	0.957
miR-133a	MIMAT0000427	0.27 ± 0.09	0.14 (0.06-1.49)	0.249 ± 0.026	0.18 (0.03-0.80)	1.10	0.419
miR-425-5p	MIMAT0003393	0.66 ± 0.161	0.38 (0.13-2.67)	0.367 ± 0.032	0.28 (0.11-1.18)	-1.45	0.120
miR-630	MIMAT0003299	1.60E-06 ±0.4E-06	1.54E-06 (0.2E-06 – 8.20E-06)	1.49E-06 ±0.39E-06	0.88E-06 (0.18E-06 – 18E-06)	-1.18	0.248
miR-625-3p	MIMAT0004808	0.20 ± 0.04	0.141 (0.05-0.82)	0.215 ± 0.035	0.13 (0.045-1.51)	1.02	0.962
miR-145-3p	MIMAT0004601	6.64E-05 ± 1.19E-05	4.03E-05 (1.26E-05-16.88E-05)	39.57E-05 ± 33.11E-05	4.37E-05 (1.31E-05 – 1529.02E-05)	1.17	0.633

* For each miRNA values are presented as Relative expression, mean ± SEM and median (range). Fold change represents a relative fold change of endometriosis group compared to disease-free controls. Statistically significant differences between the disease-free controls and endometriosis were determined using Mann-Whitney U test; the level of significance was determined as $p < 0.05$.

7.3.2.5.2 MiRNAs and the severity of endometriosis

The expression levels did not differ between rASRM stages I and II and between rASRM stages III and IV for any of the investigated miRNAs (data not shown), suggesting that the rASRM stages could be pooled together into larger subgroups: minimal-mild disease (stages I-II, n = 34) and moderate-severe disease (stages III-IV, n = 15).

Between the three subgroups (disease-free, minimal-mild and moderate-severe) there was a statistically significant difference in the expression of the three miRNAs miR-155, miR-574-3p and miR-30c (Table 7-8). MiR-155 and miR-574-3p were down-regulated in the minimal-mild forms of the disease by an average fold change of 1.5 ($p = 0.003$, $p = 0.009$, respectively). The differential expression of both miR-155 and miR-574-3p was more notable between disease-free controls and mild-minimal forms of endometriosis, than it was between disease-free controls and moderate-severe forms of endometriosis (FC = 1.25, $p = 0.286$ and FC = 1.21, $p = 0.515$, respectively). Indeed, miR-155 was down-regulated by 1.5 fold or more in 53% and by 2 fold or more in 29% of women with minimal-mild endometriosis, compared to only 27% and 13%, respectively, in women with moderate-severe disease. An analogous pattern was

observed for miR-574-3p, with a 1.5 fold or more decrease in 56% and 2 fold or more decrease in 29% of the women with minimal-mild disease, compared to 27% and 20% in women with moderate-severe forms. MiR-155 and miR-574-3p showed mild up-regulation in more severe forms of the disease, but this difference did not reach statistical significance between the groups of minimal-mild and moderate-severe endometriosis. In general, the expression of miR-155 and miR-574-3p exhibited a similar trend, with plasma levels demonstrating a positive Spearman rank correlation ($r_s = 0.65$, $p < 0.005$).

MiR-425 was down-regulated in the endometriosis group by 1.45 fold compared to the disease-free group, exhibiting reduced expression levels in both mild and severe forms of the disease by 1.5 and 1.4 fold, respectively. However, none of these changes appeared to be statistically significant, which can be explained by largely dispersed data with the highest measurements of variability for miR-425 compared to the remainder of the investigated miRNAs.

The expression levels of miR-30c and miR-133a in minimal-mild endometriosis were comparable to those in the disease-free group (FC = -1.05, p = 0.413 and FC = -1.05, p = 0.977, respectively) but exhibited up-regulation in moderate-severe forms (FC = 1.2, p = 0.089 and FC = 1.5, p = 0.03, respectively). These expression changes reached statistical significance only in comparison between mild and severe forms of the disease for miR-30c (FC = 1.23, p = 0.001) and showed a trend for significance in a similar comparison for miR-133a (and FC = 1.6, p = 0.07).

Table 7-8 Expression of the 8 candidate miRNAs set by severity of endometriosis; study phase III*

miRNA	minimal-mild endometriosis (rASRM I-II), n = 34		moderate-severe endometriosis (rASRM III-IV), n = 15		p-value[1]	FC[2]	p-value[2]	FC[3]	p-value[3]	FC[4]	p-value[4]
	Mean ± SEM	Median (range)	Mean ± SEM	Median (range)							
miR-155	0.07 ± 0.004	0.06 (0.02-0.13)	0.08 ± 0.01	0.08 (0.03-0.13)	0.009	-1.53	0.003	-1.25	0.286	1.22	0.107
miR-574-3p	0.19 ± 0.02	0.17 (0.08-0.53)	0.24 ± 0.02	0.23 (0.13-0.38)	0.015	-1.56	0.009	-1.21	0.515	1.29	0.042
miR-30c	0.59 ± 0.02	0.58 (0.35-0. 78)	0.73 ± 0.04	0.73 (0.42-0.97)	0.010	-1.05	0.413	1.17	0.089	1.23	0.001
miR-133a	0.22 ± 0.03	0.14 (0.03-0.80)	0.31 ± 0.04	0.31 (0.05-0.54)	0.095	-1.05	0.977	1.54	0.030	1.62	0.070
miR-425	0.37 ± 0.041	0.28 (0.11-1.18)	0.37 ± 0.05	0.33 (0.14-0.78)	0.262	-1.49	0.109	-1.37	0.354	1.09	0.629
miR-630	0.16E-05 ± 0.06E-05	0.91E-06 (0.18E-06 – 18.01E-06)	0.13E-05 ± 0.03E-05)	0.83E-06 (0.4E-06 – 5.33E-06)	0.476	-1.20	0.273	-1.12	0.391	1.08	0.660
miR-625-3p	0.24 ± 0.05	0.12 (0.05-1.51)	0.16 ± 0.02	0.13 (0.07- 0.29)	0.998	1.06	0.985	-1.06	0.945	0.90 (-1.12)	1.000
miR-145-3p	0.53E-03 ± 0.47E-03	0.41E-04 (0.13E-04 – 1.52E-04)	0.78E-04 ± 0.18E-04	0.52E-04 (0.17E-04 – 2.34E-04)	0.608	1.15	0.807	1.23	0.461	1.07	0.327

*The data for each miRNA are presented as relative expression, mean ± SEM and median (range). Statistically significant differences between the disease-free controls and the subgroups of endometriosis stratified by the severity of the disease were determined using Kruskal-Wallis test (statistical significance is defined as $p < 0.05$), followed by post hoc tests using Mann-Whitney U test with Bonferroni correction. The level of significance for post hoc tests was defined as $p < 0.017$ (0.05/3 [number of comparisons]);

[1] Kruskal-Wallis test;

[2] rASRM I-II vs. controls;

[3] rASRM III-IV vs. controls;

[4] rASRM I-II vs. rASRM III-IV.

7.3.2.5.3 MiRNAs and endometrioma

Ovarian involvement with endometrioma was detected in more than half of women with moderate-severe disease in our cohort (n = 8, 53%). In the subgroup analyses stratified by the presence of endometrioma, both miR-30c and miR-133a exhibited up-regulation in the presence of endometrioma. Women with endometrioma had higher expression of miR-30c and miR-133a than disease-free controls (FC = 1.25, p = 0.034 and FC = 1.96, p = 0.004, respectively) and minimal-mild endometriosis group (FC = 1.31, p < 0.001 and FC = 2.01, p = 0.024, respectively). In contrast, the expression of miR-30c and miR-133a in women with moderate-severe endometriosis without endometrioma did not differ significantly from the disease-free (FC = 1.19, p = 0.611 and FC = 1.17, p = 0.574, respectively) or minimal-mild endometriosis groups (FC = 1.15, p = 0.144 and FC = 1.23, p = 0.647, respectively) (Figure 7.1). There were no expression differences for any other of the investigated miRNAs (data not shown).

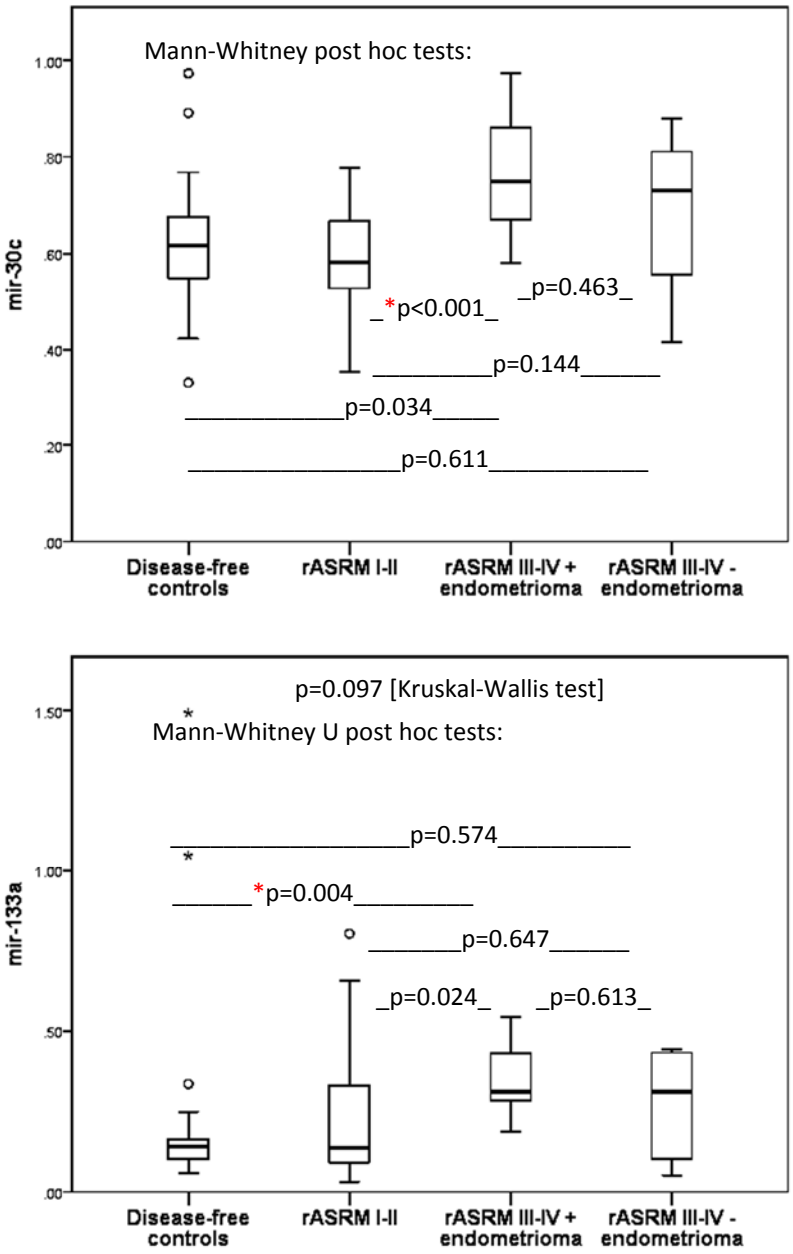


Figure 7.1 Expression levels of the 2 candidate miRNAs stratified by severity of endometriosis and by presence of endometrioma. Number of subjects per group: controls n = 19, endometriosis n = 49, rASRM I-II n = 34, rASRM III-IV n = 15, rASRM III-IV with endometrioma n = 8, rASRM III-IV without endometrioma n = 7. Box and whiskers plots represent expression levels of the candidate miRNAs. The lines inside the boxes denote the medians. The boxes mark the interval between the 25th and 75th percentiles. The whiskers denote the highest and lowest values that are not outliers or extreme values. Statistically significant differences between the disease-free controls and the stages of endometriosis were determined using Kruskal-Wallis test ($p < 0.05$ considered as significant), followed by post hoc tests using Mann-Whitney U test with Bonferroni correction ($p < 0.008$ (0.05/6 [number of comparisons] defined significance). * indicates statistically significant differences.

7.3.2.5.4 MiRNAs and other pathologic conditions

In total there were 16 women with pelvic pathology [8 (16%) women in the endometriosis and 8 (42%) women in the disease-free group] as presented in Table 7-3. Uterine fibroids were detected in 3 (6.1%) women from the endometriosis group and in 2 (10.5%) women from the disease-free group; hydrosalpinges were noticed in 1 (2%) woman from the endometriosis and in 1 (5.3%) woman from the disease-free group; CIN III was diagnosed in 1 (2%) woman from the endometriosis group based on a cervical biopsy obtained at surgery. Additionally, several women in the cohort [3 (6%) in the endometriosis group and 6 (32%) in the disease-free group were noted to have an enlarged bulky uterus, that could be suggestive of adenomyosis or intramural fibroids. The histological diagnosis of adenomyosis was obtained for 2 women from the endometriosis group who underwent hysterectomy. Unfortunately, we were not able to compare the expression levels of the investigated miRNAs between women with and without pelvic pathology because of the small sample size of the pelvic pathology group. However, the exclusion of these clinical subsets did not alter the findings obtained in the analysis of the whole cohort.

The impact of other co-morbidities on the data was then explored. Five (10.2%) women from the endometriosis group and 1 (5.3%) woman from the disease-free group were previously diagnosed with PCOS based on their menstrual pattern, signs of hyper-androgenism and/or ovarian morphology. Endocrine and metabolic states were not specifically assessed in this study. Nine (18.4%) women from the endometriosis group and 6 (32%) women from the disease-free controls suffered from chronic medical conditions such as asthma, diabetes mellitus, hypothyroidism, rheumatoid arthritis, cardiomyopathy, irritable bowel syndrome, and mood disorders. Women with PCOS and women who suffered from chronic medical conditions were excluded in separate sub-analyses. The exclusion of the subsets with PCOS did not change the differential expression pattern for miR-155 ($p = 0.005$) and miR-574-3p ($p = 0.027$). In the analysis limited to the subsets without any chronic medical conditions (12 women in the disease-free group and 38 women in the endometriosis group), miR-155 demonstrated an expression profile comparable to that detected in a whole cohort with a significant difference between endometriosis and disease-free groups ($p = 0.020$). Although the median expression of miR-574-3p remained lower in endometriosis (0.172 (0.081-0.535) vs. 0.234 (0.122-0.703) in disease-free group), its differential expression did not appear significant ($p = 0.091$).

7.3.2.5.5 MiRNAs and recurrent endometriosis

Fourteen (29.2%) women from the endometriosis group had a previous laparoscopy for pelvic pain with identification and surgical treatment of endometriotic lesions. No significant difference was observed in the levels of any of the investigated miRNAs between the endometriosis patients with and without previous surgically treated endometriosis, i.e. with and without recurrent disease.

7.3.2.5.6 MiRNAs and endometriosis in the past

Ten (55%) of the disease-free women had had a previous laparoscopy for pelvic pain. Endometriosis was surgically diagnosed and treated in 4 (22%) women, whilst the absence of the disease was surgically confirmed in 6 (33%). In addition, some of the disease-free women had pelvic findings suggestive of endometriosis in the past, which included peritoneal scarring noted in 3 women and severe adhesions in 1 woman. Three out of these 4 women had had a laparoscopy in the past for investigation and treatment of pelvic pain, with surgically confirmed endometriosis in 1 woman. To evaluate the homogeneity of our disease-free group, we performed comparative analyses after exclusion of the subsets with pelvic findings from the disease-free group. The patterns of expression and the levels of significance concerning candidate miRNAs did not differ from the results obtained on the whole group of controls. The analysis performed after exclusion of the additional 3 women with previous surgical diagnosis of endometriosis and currently normal pelvis from the disease-free group revealed similar results. The subgroup analyses within the disease-free women classified by presence or absence of endometriosis in the past were not performed due to the limited sample size in each subgroup.

7.3.2.5.7 MiRNAs and hormonal treatment

Nineteen (39.6%) women from the endometriosis group and 2 (10.5%) women from the disease-free group were treated with hormonal medications at the time of blood collection, predominantly the combined oestrogen – progesterone contraceptive pill. A comparison of the investigated miRNAs expression between women who did and did not receive hormonal therapy separately within endometriosis and disease-free groups showed no statistically significant difference. However this comparison was meaningful only in the endometriosis group due to the limited sample size in the disease-free subgroups of patients treated and not treated with hormonal medications (n = 2 and n = 17, respectively). Exclusion of the subsets using hormonal treatment from both endometriosis and disease-free groups did not alter the

differential expression and the significance levels for any of the investigated miRNAs except miR-133a. The expression of miR-133a was decreased in subsets on hormonal treatment and appeared to be significantly up-regulated in moderate-severe endometriosis compared to either disease-free or to minimal-mild endometriosis groups when only subsets without hormonal treatment were analysed. However, according to Spearman's correlation coefficient, no significant correlation was found between the hormonal treatment and the expression of miR-133 in the endometriosis (r_s = -0.409, p = 0.092) or disease-free group (r_s = -0.166, p = 0.255) or in the overall cohort (r_s = -0.166, p = 0.178). No correlation was identified between the hormonal treatment and the remainder of the investigated miRNAs.

7.3.2.5.8 MiRNAs and the phases of the menstrual cycle

Twenty five women in the endometriosis group and 15 women in the disease-free group had regular menstrual cycles and were not using contraceptive hormonal medications. The cycle length for the majority of the women in our cohort was 26-30 days, with the exception of 5 women (2 from the endometriosis and 3 from disease-free group) who had a 21 day cycle. Women were classified by the phases of the cycle as following: menstrual phase (1 woman with endometriosis and 2 disease-free women), proliferative phase (10 women with endometriosis and 3 disease-free women), peri-ovulatory phase (7 women with endometriosis) and luteal phase (7 women from each group). There was no change in the levels of the investigated miRNAs across the phases of the menstrual cycle in the endometriosis group. Unfortunately, analyses concerning phases of the cycle in disease-free group were not possible because of the limited sample size in the subgroups.

7.3.2.6 Diagnostic accuracy of the candidate miRNAs

ROC curve analyses revealed that both miR-155 and miR-574-3p are potential biomarkers for endometriosis detection, resulting in AUC (area under the ROC curve) = 0.705, (95% CI, 0.575-0.835), p = 0.009, and AUC = 0.67, (95% CI, 0.524-0.816), p = 0.03, respectively (Figure 7.2 and Figure 7.3). The optimal sensitivity, specificity and overall percentage of accuracy using the probability cut-off of 0.5 were 95.9%, 26.3% and 76.5% for miR-155 and 98%, 26.3% and 77.9% for miR-574-3p, respectively.

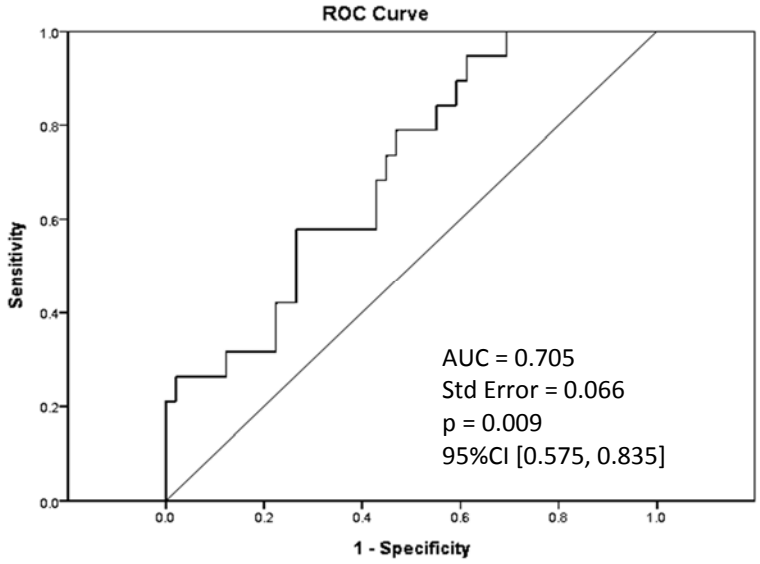


Figure 7.2 ROC analysis using plasma miR-155.

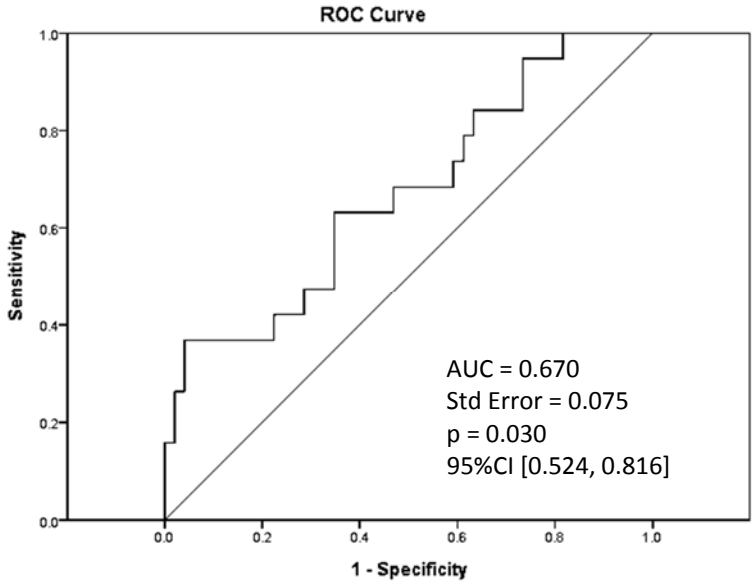


Figure 7.3 ROC analysis using plasma miR-574-3p.

At the cut off value of 0.060 for miR-155, the sensitivity was 84% and the specificity was 45%. At the cut off value of 0.162 for miR-574-3), the sensitivity was 84% and the specificity was 37%. The odds ratio for miR-155 being associated with endometriosis was 0.805 (95% CI, 0.684-0.946) and for miR-574-3p the odds ratio was 0.942 (95% CI, 0.901-0.985) for each 0.01 point decrease on the scale of miRNA level.

An addition of miR-574-3p could not improve the differentiation between women with and without disease (AUC = 0.701, (95%CI, 0.569-0.834)), indicating an overlap between the diagnostic values of miR-155 and miR-574-3p (Figure 7-4).

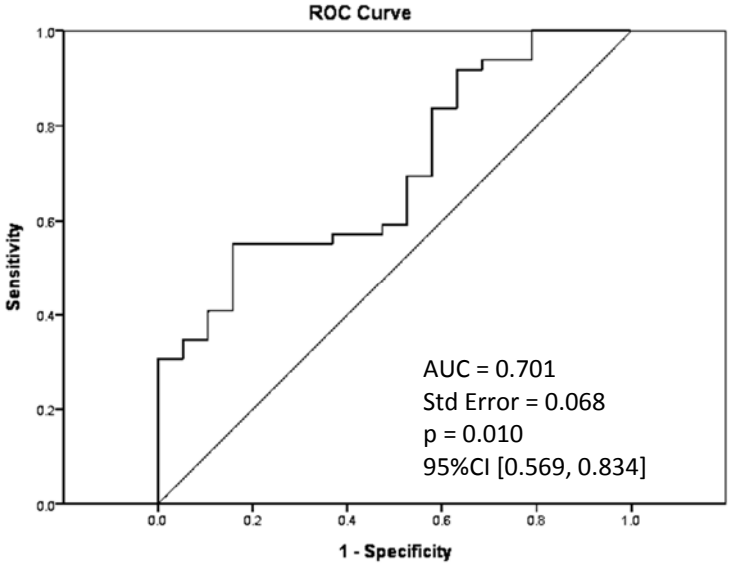


Figure 7-4 ROC analysis for the combination of plasma miR-155 + miR-574-3p

Interestingly, incorporation of miR-133a and miR-30b into a predictive model improved the discriminatory power of miR-155 and miR-574-3p to distinguish the endometriosis and disease-free groups. Analyses of the ROC curves for plasma miRNAs showed that the AUC was greatest for the combination of miR-574-3p, miR-133a and miR-30c resulting in AUC = 0.774, (95% CI, 0.656-0.893) with 98% sensitivity, 33% specificity and 80.6% of overall accuracy with a cut off of predicted probability at 0.5, indicating the additive effect in the diagnostic value of these 3 miRNAs (Figure 7.5).

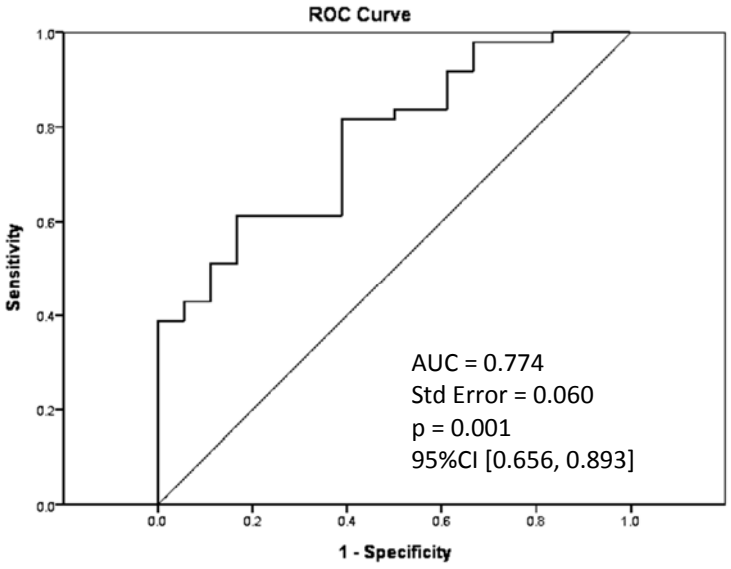


Figure 7.5 ROC analysis of regression model using miR-574-3p, miR-133a, miR-30c to discriminate endometriosis.

Next, we examined a predictive model incorporating clinical parameters combined with the identified miRNAs. With the univariable logistic regression model, infertility, history of miscarriage and number of the past miscarriages was significantly correlated with endometriosis. Spearman rank order coefficient suggested only a weak negative relationship with endometriosis for each of the abovementioned parameters, as following: infertility (r_s = -0.278, p = 0.026), history of miscarriage (r_s = -0.258, p = 0.038), and number of past miscarriages (r_s = -0.311, p = 0.012). No significant correlation was found between endometriosis and the rest of the demographic and clinical characteristics, such as age, BMI, smoking, parity, regularity of the menstrual cycle, migraine, abnormal uterine bleeding, duration of symptoms and a family history of endometriosis (Table 7-9). Using multivariate logistic regression, several predictive models were created (Table 7-10). A predictive model that included parameters such as infertility, previous miscarriages and the expression of miR-155, miR-574-3p, miR-133a and miR-30c demonstrated an overall accuracy of 84.1% with 93.5% sensitivity, 58.8% specificity and AUC = 0.831, (95% CI, 0.719-0.943), p < 0.001 (Figure 7.6)

Table 7-9 Univariate logistic regression model for association of clinical parameters with endometriosis

	B[1]	SE[2]	Wald[3]	p-value[4]	Exp (B)[5]	95% CI for Exp (B)[6]
Infertility	1.281	0.592	4.688	0.030	3.600	1.129, 11.478
Previous miscarriage	1.217	0.602	4.088	0.043	3.378	1.038, 10.992
Number of previous miscarriages	-.705	0.324	4.750	0.029	0.494	0.262, 0.931
Age	-.055	0.031	3.292	0.070	0.946	0.891, 1.004
BMI	-0.033	0.053	0.397	0.528	0.967	0.872, 1.073
Smoking	1.006	0.747	1.813	0.178	2.733	0.632, 11.813
Parity	-0.582	0.307	3.587	0.058	0.559	0.306, 1.020
Pattern of the menstrual cycle	0.094	0.750	0.016	0.901	1.098	0.252, 4.780
Migraine	0.405	0.563	0.519	0.471	1.500	0.498, 4.519
Uterine bleeding	0.642	0.613	1.095	0.295	1.900	0.571, 6.323
Duration of the symptoms	0.039	0.038	1.066	0.302	0.961	0.892, 1.036
Family history of endometriosis	-0.247	0.659	0.141	0.708	0.781	0.215, 2.841

[1] **B** – the coefficients for logistic regression equation for predicting the dependent variable from the independent variable (log-odds units); the prediction equation is: log(p/1-p) = b0 + b1*x1 + b2*x2 + b3*x3 + b4*x4);

[2] **SE** – standard error around the coefficients;

[3] **Wald** – Wald chi-square test that tests the null hypothesis that the coefficient (parameter) equals 0;

[4] **p-value** – significance of the Wald test;

[5] **Exp (B)** – the odds ratios for the predictors;

[6] **CI** – confidence interval.

Table 7-10 Comparison of predictive models for endometriosis identified with multivariate logistic regression analyses

Model	Omnibus test of model coefficients			Model summary			H-L test	Classification table			AUC (95%, CI)
	X^2	df	p	-2 LL	Ra^2	Rb^2		Sensitivity	Specificity	Overall accuracy	
Previous miscarriages, infertility, miR-155, miR-574-3p, miR-133a, miR-30c	22.435	6	0.001	51.036	0.300	0.435	0.929	93.5%	58.8%	84.1%	0.830 (0.719, 0.941)
Previous miscarriages, infertility, miR-155, miR-574-3p, miR-30c	18.886	5	0.002	57.163	0.256	0.368	0.632	95.7%	44.4%	81.3%	0.785 (0.653, 0.917)
Previous miscarriages, infertility, miR-155, miR-574-3p, miR-133a	22.326	5	<0.001	51.145	0.298	0.433	0.176	93.5%	58.8%	84.1%	0.831 (0.719, 0.943)
Previous miscarriages, infertility, miR-155, miR-574-3p	18.768	4	0.001	57.281	0.254	0.366	0.780	95.7%	44.4%	81.3%	0.796 (0.668, 0.923)
Previous miscarriages, infertility, miR-574-3p, miR-133a	19.917	4	0.001	55.553	0.271	0.394	0.708	93.5%	58.8%	84.1%	0.809 (0.686, 0.933)
Previous miscarriages, infertility, miR-155, miR-133a	18,682	4	0.001	54.789	0.257	0.373	0.313	93.55	35.3%	77.8%	0.825 (0.715, 0.935)
Previous miscarriage, miR-155, miR-574-3p, miR-133a	18.513	4	0.001	55.581	0.251	0.366	0.667	95.7%	41.2%	81.3%	0.810 (0.692, 0.927)
Previous miscarriage, miR-574-3p, miR-133a	16.362	3	0.001	57.462	0.229	0.334	0.536	93.6%	41.2%	79.7%	0.787 (0.660, 0.915)
Previous miscarriage, miR-155, miR-574-3p	15.804	3	0.001	60.899	0.216	0.312	0.385	97.5%	27.8%	78.5%	0.771 (0.640, 0.901)
Infertility, miR-155, miR-574-3p, miR-133a, miR-30c	18.226	5	0.003	55.245	0.251	0.365	0.376	95.7%	35.3%	79.4%	0.824 (0.709, 0.938)
Infertility, miR-155, miR-574-3p, miR-30c	15.630	4	0.004	60.419	0.217	0.312	0.377	97.8%	33.3%	79.7%	0.762 (0.629, 0.896)
Infertility, miR-155, miR-574-3p, miR-133a	18.048	4	0.001	55.423	0.249	0.362	0.504	95.7%	35.3%	79.4%	0.812 (0.695, 0.929)

X^2 – chi –square statistics;

df – number of degrees of freedom for the model;

p – probability of obtaining chi-square statistics given that the null hypothesis is true (no effect of the independent variables);

-2LL – 2-log likelihood for the final model (used to compare nested (reduced) models;

Ra^2 and **Rb^2** – Cox & Snell R Square and Nagelkerke R Square, respectively (the pseudo R-squares);

H-L test – Hosmer – Lemeshov test (test for goodness of fit for logistic regression models).

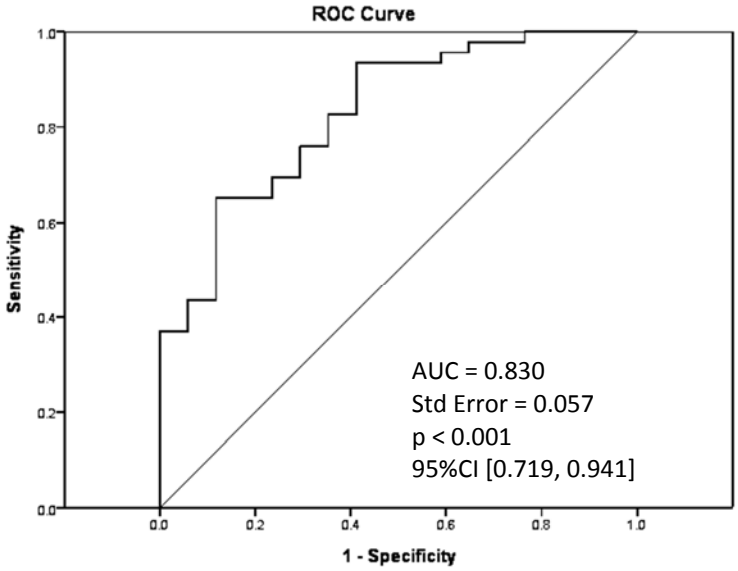


Figure 7.6 ROC analysis of regression model using the combination of clinical parameters (infertility and number of miscarriages) with miR-574-3p, miR-133a, miR-155 and miR-30c to discriminate endometriosis.

7.4 Discussion

This study was to evaluate circulating miRNAs in a clinically well-described cohort with surgically confirmed diagnosis of endometriosis. A set of 4 miRNAs displayed aberrant expression in plasma of women with endometriosis compared with disease-free women, suggesting that the measurement of miRNAs in plasma may be useful in blood based detection methods for endometriosis.

MiR-155 and miR-574-3p levels were significantly decreased in plasma from endometriosis patients. However, the lowest levels of both miRNAs were detected in women with minimal-mild disease. In moderate-severe forms, both miR-155 and miR-574-3p displayed lower concentration than that observed in disease-free women, but a higher concentration than in minimal-mild disease. A positive Spearman rank correlation was observed between miR-155 and miR-574-3p and there was a similarity in the expression pattern suggesting that these miRNAs are likely to be co-regulated in endometriosis and might share common regulation pathways.

The expression levels of two miRNAs, miR-133a and miR-30c remained unchanged in minimal-mild forms, but were elevated in the severe forms of endometriosis, suggesting that these miRNAs might reflect the severity of endometriosis. None of the demographical or clinical parameters appeared to confound the expression of the identified miRNAs.

Both linear and logistics regression analyses further demonstrated that age, BMI, smoking, parity and previous miscarriages did not influence the plasma levels of miR-155, miR-574-3p, miR-133a and miR-30c, suggesting their usefulness as potential biomarkers for a broad unselected population.

It is unclear why miRNA expression differed with different stages of endometriosis. We can hypothesise that the extent and depth of infiltration associated with severe forms of the disease result in increased cell mass in the endometriotic lesions, which could possibly lead to increased release of certain miRNAs into the circulation. It has been shown that deep endometriotic nodules more readily secrete more CA-125 and PP14 into the blood stream, whereas superficial, mild, pelvic endometriosis with less developed blood supply tends to secrete CA-125 and PP14 into the peritoneal cavity rather than into the blood (550).

Mild and severe forms of endometriosis also might represent different entities with distinct pathophysiology and thus may be associated with different miRNA profile. This is supported by previously demonstrated differences in the characteristics of peritoneal fluid (551), in the phenotype of immune cells and macrophages in peritoneum (552) and in the genome profiles of eutopic endometrium (531) between severe and mild forms of endometriosis.

We can also assume that over-expression of miR-133a and miR-30c might be a cause rather than consequence of the disease. MiR-133a, known for its functions in controlling myoblastic proliferation and differentiation, was found to be significantly expressed in mitochondria, suggestive of being involved in a mitochondrial mRNA silencing regulation (553). Mitochondria provide metabolic energy, initiate apoptosis and synthesise the steroid hormones, and it may be that miR-133a plays an important role in regulation of these events, which are important in the progression of endometriosis. MiR-30c has been reported to suppress apoptosis via inhibition of p53 expression (554). Both miRNAs, miR-133a and miR-30c, were previously found to be co-expressed in rodent models of heart disease (555).

Another possibility is that both miR-30c and miR-133a are associated specifically with endometriomas, rather than with severe endometriosis, as they were elevated in women with ovarian endometrioma. Previous reports have identified endometrioma as a distinctive clinical form compared with peritoneal disease (556). It is tempting to speculate that an endometrioma miRNA profile exists. If this is the case, concurrent miRNA testing may improve the sensitivity of imaging to discriminate endometrioma from other types of ovarian tumours. Finally, the possibility cannot be excluded that this expression pattern could be observed

simply due to random association and may not be apparent in a larger group. Further studies are required to confirm and to characterise the miRNA profile associated with different disease stages and with the presence of endometrioma.

Both miR-155 and miR-574-3p levels remained suppressed after exclusion of participants with any chronic diseases or other pelvic pathologies, although miR-574-3p lost statistical significance when only women without chronic medical conditions were included in the analysis. This presumably was related to a reduced sample size, but this study could not conclusively establish that other co-morbidities did not confound the expression of miR-574-3p. Women with malignant disease or with acute infection were excluded from the present study in order to avoid a possible bias associated with the ability of these conditions to modulate miRNA expression. The impact of different medical conditions, including malignancy and infection on circulating miRNAs associated with endometriosis requires further validation in a larger cohort.

Recurrent endometriosis and a previous diagnosis of endometriosis in currently disease-free women did not appear to affect the discriminative ability of miR-155 and miR-574-3p. It may be that both miRNAs reflect only active ongoing disease and could be valuable indicators of the recurrence or response to treatment. However, again this speculation requires further validation in a larger cohort.

Treatment with hormonal medications was not associated with altered expression of miR-155, miR-574-3p and miR-30c, but may suppress expression of miR-133a. However, the subgroup analysis was limited by the small sample size of women not taking hormonal medications. MiR-155 and miR-574-3p were not influenced by the phase of the menstrual cycle or the physiological hormonal environment.

All the dysregulated plasma miRNAs are associated with other pathologies and are not specific for endometriosis. For example, miR-155 has been demonstrated to target multiple components of the inflammatory cytokine production cascade, TLR and intracellular signalling pathways, exhibiting pro-and anti-inflammatory qualities (557-558). Circulating levels of miR-155 were significantly up-regulated in rheumatoid arthritis and in inflammation-induced liver injury (559) and down-regulated in patients with coronary artery disease (261) and various cancer types (262, 560-561). Aberrant expression of miR-574-3p has been reported in association with several malignancies and autoimmune diseases (503-507). MiR-133a is well documented in heart and lung disease as well as in various types of cancers (202, 562) and in

endometrial endometrioid adenocarcinoma (563-565). MiR-30c has been found to be up-regulated in ovarian cancer (566) and was connected to a range of human diseases including heart disease and other types of cancer (327). Therefore, a combined panel of biomarkers rather than a single biomarker is more likely to be a more specific diagnostic discriminator.

The reduced concentration of circulating miR-155 and miR-574-3p detected in patients with endometriosis was surprising, because one might expect that inflammation associated with endometriosis would induce the expression of miR-155, thereby elevating its levels. Moreover, if miRNAs were released into the systemic circulation from endometriotic tissue (248), we would anticipate that miR-574-3p would be up-regulated in plasma as it is present in high levels in endometriotic tissues (205), and it could be anticipated that miR-574-3p would be elevated in plasma in patients with endometriosis.

Notably, out of 24 miRNAs that were selected by the array experiment, only 4 were found to be significantly dysregulated in endometriosis in this validation study. Furthermore, the magnitude of the relative fold changes differed between the miRNA arrays and the singleplex qRT-PCR experiments. Such disagreement can be attributed to the differences in normalisation approaches and data analysis methods. Unexpectedly, miR-155 was found to be down-regulated in endometriotic plasma in this experiment, whereas it was noted to be up-regulated on the arrays. This suggests a possible increased sensitivity of this miRNA to differences in design between the microarray and qPCR primers, the array pre-amplification step and/or different normalisation approaches. Although some differences were seen in the 2 alternate experimental settings, both methods provided largely concordant direction of expression for the remaining tested circulating miRNAs.

Another possible explanation for discrepancy between the array and qRT-PCR experiments might be the use of different control groups in each of the experiments, i.e. a prospective cohort of symptomatic women in the qRT-PCR-based validation study, as opposed to asymptomatic healthy controls in the array experiment. It is also possible that chronic pelvic pain syndrome modulates circulating miRNAs even in the absence of endometriosis. Finally, a bias related to an incorrect diagnosis of endometriosis at surgery, particularly in minimal forms of the disease, is possible. The early stages of endometriosis have been described as a dynamic event that occurs intermittently in most women, suggesting that the presence of lesions is dynamic and can change over time (532), which could negatively impact on correct

allocation to analysis groups. Even though selection of symptomatic controls may have an impact on the differential expression of miRNAs between the groups, this is exactly the population that would need to be distinguished from those with endometriosis and a non-invasive test would be used in.

A common problem in circulating miRNA research is that no consensus has been reached regarding internal controls. Normalization is a key step for the accurate quantification of RNA levels with qRT-PCR, which is particularly relevant in diagnostic studies. In this study we established a method to reproducibly and accurately measure miRNAs in plasma, carefully selecting endogenous controls in the screening array experiment and verifying their stability by using qRT-PCR in a subset of experimental samples.

A recently published study by Wang et al., 2012, identified endometriosis-associated miRNAs in serum from 60 patients and 25 controls and suggested that a panel of 4 differentially expressed miRNAs had a 93.2% sensitivity and 96 % specificity for predicting endometriosis in a retrospective cohort (211). While this is exciting, the choice of miRNAs was based on pooled un-replicated experiments, therefore we suggest that potentially useful diagnostic miRNAs may have been missed. Endometriosis associated differential expression > 2 fold was seen in 145 miRNAs, whereas 61 demonstrated a > 10 FC difference. Of these only 1 was identified in our study (miR-145*), and we were unable to replicate these findings in our dataset when we normalised to U6 in concordance with the Wang et al. methodology.

Jia et al. performed another microRNA microarray analysis comparing plasma samples from groups of 3 women with and without endometriosis (212). Twenty seven down-regulated microRNAs were identified when the data set was normalised to a preselected endogenous control miR-16. Only one miRNA, miR-23a, was concordant with the miRNAs identified in our study, and another, miR-122, was identified by Wang et al., but was differentially expressed in the opposite direction. This inconsistency between the 3 studies could be due to inherent differences in the sample population, including geographic and ethnic differences as well as differences in patient selection criteria. For example, most control patients in the Wang et al' study suffered from infertility without pelvic pain, whereas control patients in the Jia et al. study included women with leiomyoma and ovarian masses, which raises the question whether these women truly represent the population that would be routinely tested for endometriosis using a plasma miRNA assay. Technical differences in data analysis and

normalisation are also likely to play role, which emphasises the need for further work in the plasma miRNA diagnostic field, and especially for studies involving larger numbers of women.

It is clear that normalisation is a critical step in data analysis and different methods can lead large variations in outcomes using the same dataset (205-209, 240-241). Wang et al.'s study was unable to use a data-driven approach to select an optimal normalisation method since their pooled study had no replicates, and therefore a default normalisation approach of U6 was used. However, others have found that U6 is not a reliable endogenous control (31) and this proved true in our dataset, where RNU-6B, a small nuclear non coding RNA also known as U6, appeared to be differentially expressed between endometriotic and control patients. Other studies demonstrated that RNU-6B does not fulfil criteria for endogenous controls and should be abandoned as a normaliser in miRNA qRT-PCR experiments (567-568). The use of standard single reference genes like U6 and miR-16 is increasingly difficult to support given the frequently observed variation of standard genes across experimental conditions (569). Quantile normalisation has proved the most effective normalisation strategy for this current study, in accord with previous evaluations by others (301). However this approach is less effective when only small numbers of miRNAs are assayed, as is likely to occur as clinical plasma miRNA assays are developed and tested. Therefore, plasma miRNA normalisation strategies based on miRNAs specific to the experimental sample set, or assays based on complex ratios between multiple miRNAs will need to be developed in order to develop robust plasma miRNA assays for endometriosis.

All 3 studies, however, revealed a distinct miRNA expression pattern in the circulation of endometriosis sufferers indicating that further exploration is required in this field. To date, no study has assessed the diagnostic value of circulating miRNAs in a test cohort which is a crucial evaluation of the robustness of a potential diagnostic test.

The diagnostic accuracy estimates presented in this thesis suggest that a plasma miRNA-based predictive model is no more accurate than surgical diagnosis, given moderate specificity of 58.8%. However, the high sensitivity of 93.5% suggests the possibility of using this model as a triage test. A triage test would permit a more thorough selection of candidates for surgery and provide an opportunity to intervene at an earlier time. Although a non-invasive test with high sensitivity and specificity would be ideal, from a clinical perspective it is important to be able to screen large numbers of symptomatic women in a less invasive, less expensive and relatively simple way. It is important that women with endometriosis who might benefit from

early surgery are not missed by the test. Therefore, a triage test for endometriosis is required to have a higher sensitivity, with a low number of false-negative results, i.e. a low number of patients who have a negative test and who do have endometriosis. This test might have a lower specificity, which implies a higher number of false-positive results, i.e. a higher number of patients who have a positive test but who do not have endometriosis requiring surgery. Without higher specificity, there would be women who would undergo unnecessary surgery, but the number of surgeries on women without endometriosis would be less than it would be without test. Further studies are needed to validate and address the potential diagnostic role of miRNA profiles in endometriosis, including in combination with other types of biomarkers, and their possible use in stratifying patients with respect to clinical parameters and severity of the disease. Moreover, profiling plasma miRNA expression before and after surgical treatment for endometriosis could also assess the correlation between plasma miRNA profiles and tissue load and determine if plasma miRNAs had potential as markers of disease progression. We aim to recruit women for this type of study in the near future.

7.5 Limitations

It should be noted that consideration of circulating miRNAs as biomarkers for endometriosis is at present based on our results from a relatively small sample size and larger clinical studies are clearly required to establish the case. As described in a sentinel review on this subject by Yang and Speed (351), it is difficult to estimate the number of patients needed for this kind of follow-on genomics study. Our experience suggests that plasma miRNA expression values may in general have more variability than tissue miRNA expression levels. If we use the median variance of the differentially expressed miRNAs within our TaqMan array data set, power calculations suggest that over 150 patients in each of the control and endometriotic groups may be required to have a 90% chance of detecting a true two-fold difference in plasma miRNA abundance between the groups. The recruitment of subjects for these types of studies is challenging. We were not able to recruit sufficient amount of subjects (above 300) to fully comply with the above power calculation due to substantial financial and time constrains. Given that, we acknowledge that further investigation in this field with larger amount of subsets in each subgroup is mandatory and we are undertaking a larger study as a part of commercial development of miRNA-based product in order to validate the findings presented in this thesis.

Furthermore, 24 miRNAs carefully selected with the array experiment were initially validated only in 20 subsets. This may result in underestimation of the discriminative ability of some of the miRNAs that were not chosen for validation in the remained samples. Moreover, we initially screened for 674 miRNAs, whilst the majority of the circulating miRNAs remain untested and we can make no claims about their association with endometriosis. Finally, we cannot provide molecular insights into the cause of the dysregulation. The levels of circulating miRNAs may be affected by multiple parameters, ranging from the multifactorial pathogenetic mechanisms to the stability of miRNAs in plasma.

7.6 Conclusions

The results of the current study present potential novel biomarkers for early detection of endometriosis. Our patient and control groups are not sufficiently large to claim that we have characterized a new biomarker, but the present study does lay the groundwork for future efforts to identify and develop miRNA blood-based biomarkers for endometriosis. Future studies with broader miRNA profiling may reveal additional aetiology-specific miRNA signatures and would also investigate the relationship between the miRNAs and the disease severity.

Chapter 8
General discussion and conclusions

8.1 Introduction

Endometriosis is a common disabling disease of young women, with significant morbidities and costs associated with diagnosis and treatment. The invasive nature of the current surgical diagnostic method is associated with a substantial diagnostic delay and limits our ability to research the effectiveness of non-surgical therapies. Our lack of understanding of the cellular mechanisms of endometriosis limits our ability to identify biomarkers with the potential to diagnose the disease non-invasively. A growing body of evidence suggests that endometriosis affects multiple organ systems, which could be the due to systemic alterations in women with endometriosis. Identifying unique circulatory signatures of endometriosis could result in a better understanding of the disease as well as the development of a non-invasive diagnostic test.

MiRNAs regulate important cellular processes via fine tuning of gene networks and appear to be a powerful tool in biomedical research. In particular, miRNA signatures detected in plasma and serum have been proposed as diagnostic markers in various diseases. The advantage of using miRNAs as biomarkers is that the traditional protein-based biomarker discovery pipelines often require a priori knowledge of the biomarker candidates, whereas currently available high throughput miRNA technologies facilitates the simultaneous screening of hundreds of miRNAs. This increases the efficiency of the identification of disease-specific panels of dysregulated miRNAs as well as their gene and protein targets. Furthermore, miRNAs can be precisely quantified using qRT-PCR that provides exceptionally high sensitivity and specificity of detection and also allows absolute quantification of miRNAs down to the level of copy number per RNA sample, potentially providing precise cut-off concentrations for the purpose of diagnosis.

The work presented in this thesis centres on the expression of plasma miRNAs in endometriosis, addressing the relevance of these miRNAs in pathophysiology of the disease, and their correlation with expression in eutopic endometrium. The endpoint of the project was to assess the utility of plasma miRNAs for diagnosis of endometriosis. At the commencement of this project, miRNA profiles have been identified in eutopic and ectopic endometrium in endometriosis, but there were no data on the expression of miRNAs in the systemic circulation in association with this disease.

A step-wise approach was undertaken in attempting to investigate the expression of circulating miRNAs in endometriosis. Firstly, we developed a methodology for reliable and reproducible quantification of circulating miRNAs (Chapter 3), based on a detailed analysis of the existing knowledge in the field (Chapter 2). Secondly, in order to assess influence of physiological fluctuations of ovarian steroids on miRNA expression in reproductive-age women, we screened for miRNA expression across the different phases of menstrual cycle in a healthy population and in women with endometriosis (Chapter 4). Thirdly, we identified an endometriosis-specific miRNA profile and demonstrated a concordance between the micro array results and singleplex qRT-PCR, which is the gold standard for miRNA detection (Chapter 4). Next, in order to assess functional relevance of the identified miRNAs in endometriosis, we performed *in silico* functional analyses of the predicted targets of dysregulated miRNAs (Chapter 4) and summarised the literature on the functional involvement of these miRNAs in biological processes similar to those known to occur in endometriosis (Chapter 5). In order to identify a relationship between miRNAs in the circulation and in the eutopic endometrium in endometriosis, we identified the eutopic endometrial miRNA profile in women with and without endometriosis (Chapter 6). Finally, we conducted a prospective diagnostic accuracy study on a larger cohort to assess the validity of circulating miRNAs for diagnosis of endometriosis (Chapter 7).

8.2 Quantification of circulating miRNA and approach to the data analysis

MiRNA research is a relatively new field of molecular biology and there is no consensus regarding the measurement and analysis of miRNA data, particularly with respect to low abundant circulating miRNAs (Chapter 2). It is clear that different methodological approaches alter the experimental results and can have significant impact on the final conclusions. In this project we established a method to reproducibly and accurately measure miRNAs in plasma (Chapter 3). Normalization is a key step for the accurate quantification of RNA levels with qRT-PCR and we deduced that quantile normalisation is optimal for large-scale experiments, whereas singleplex qRT-PCR quantification requires endogenous controls that should be carefully selected for the experimental dataset. RNU6B, a member of small RNA U6 family has been previously suggested as a stable endogenous control, however in our datasets it was not stable between the groups in our experiments (Chapter 4), which led to its exclusion as a control. Additionally, we explored the exclusion of “low call detectors” as a process to obtain

more meaningful diagnostic results. This strategy of miRNA identification (Chapter 3) was an important step in development of the technique to measure miRNAs from plasma and/or serum with sufficient sensitivity and precision to be clinically effective.

8.3 Influence of the menstrual cycle on expression of circulating miRNAs

Cyclical changes in reproductive steroids appeared to have little impact on plasma miRNA profiles in either healthy control or endometriotic populations. Our data indicates that plasma diagnostic tests based on the miRNAs identified as disease-specific are likely to be accurate at any time in the menstrual cycle in reproductive aged women. This information is of particularly important in biomarker research, where the stage of the menstrual cycle in which blood is drawn, or the use of the hormonal medications, might have a negative impact on the performance of a miRNA-based test. The absence of significant menstrual phase variations in plasma miRNA expression does not exclude the existence of miRNA-hormonal interactions nor does it contradict the previous reports of cycle-dependant miRNA pattern in endometrial cells and tissue, in which high local concentration of reproductive steroid hormones are likely to have a greater impact on miRNA profiles.

8.4 Plasma miRNA profile in endometriosis

We demonstrated a distinct miRNA expression pattern in the plasma from endometriosis sufferers (Chapter 4). Six plasma miRNAs were up-regulated and 6 down-regulated in endometriosis compared to healthy controls whereas 45 plasma miRNAs showed a trend to significant differential regulation in endometriosis. This suggests there is considerable potential to develop a non-invasive diagnostic test using plasma miRNAs.

Although we did not find menstrual cycle dependent miRNA expression levels in either the control or endometriosis groups, 7 of the 12 miRNA transcripts were able to distinguish women with and without endometriosis only at certain phases of the cycle. The late proliferative stage appeared to be the best sampling phase with 10 of the miRNAs demonstrating an ability to distinguish women with endometriosis from healthy controls. It may be that women with endometriosis have a mild divergence from the usual physiological responsiveness to steroid hormones in the late follicular phase that is apparent only when women with and without endometriosis are compared. This could explain why women with

endometriosis tend to have a prolonged proliferative phase and are resistant to P4 compared to healthy controls.

Few miRNAs were dysregulated in both plasma (in our study) and endometriotic lesions (published data from other groups) and all of the miRNAs that were concordant in both compartments were down-regulated in plasma. It is therefore unlikely that the direct excretion of miRNAs from endometriotic tissues into the circulation accounts for the presence of endometriosis-associated miRNAs. Even though the origin of endometriosis-associated circulating miRNAs remains unknown, this observation supports an assumption that miRNAs are actively secreted from the cells, rather than passively released from the damaged endometriotic cells.

8.5 Functional involvement of dysregulated plasma miRNAs in endometriosis

Functional analyses of endometriosis-associated miRNAs and their predicted targets allowed identification of mRNA targets, biological and functional pathways and molecular networks that demonstrate concordance with our current knowledge of the cellular and molecular events associated with endometriosis (Chapter 4). The top functional networks converged on CTNNB1, which is a key factor in the Wnt, MAPK and NFkB signalling and on its downstream target CCND1. Additional molecular networks constituted by the endometriosis-associated predicted targets converged on molecules involved in inflammation (NFkB), proliferation (MAPK), tissue remodelling (TGF-β) and steroidogenesis (ER, PR, LH, FSH). These functional analyses raise the possibility that plasma miRNAs may provoke some of the adverse health epiphenomenon associated with endometriosis. If this was validated in further study, therapeutic alteration of endometriosis-associated plasma miRNAs levels might have potential utility to reduce the risk of health sequelae from endometriotic disease.

Even though most of the deregulated plasma miRNAs were not directly linked to endometriosis, their aberrant expression has been described in the context of inflammatory, auto-immune and malignant diseases (Chapter 5). Functional associations and regulatory mechanisms indicate that TP-53, TGF-β, MAPK, NFkB and Wnt-signalling pathways are likely to modulate the expression of the miRNAs identified in our study. For most of the investigated miRNAs, the direction of dysregulation varies in different types of medical conditions, suggesting a tissue- or disease-specific action. Collectively, our results further highlight the

putative role of miRNAs in the pathogenesis of endometriosis as their main biological functions linked to endometriotic disease.

8.6 MiRNA expression in eutopic endometrium in endometriosis

Array based global miRNA profiling in secretory eutopic endometrium, including both endometrial and stromal compartments, revealed differentially expressed miRNAs in women with endometriosis (Chapter 6). Our results did not correlate with previously published data, which could be explained by differences in samples processing, in detection platforms as well as by differences in the endometriosis and control subsets between the studies. The comparison between women with pelvic pain ± infertility with and without endometriosis revealed 2 differentially expressed miRNAs, miR-551a and miR-148a*. It is possible that these miRNAs could become endometrial biomarkers for endometriosis; however an endometrial biopsy either solely or in combination with the blood test would be required.

The miRNA profile was different when disease-free women with pelvic pain were compared to those without pain, suggesting that chronic pain alters the eutopic endometrium irrespective of endometriosis. It might be possible that the neural markers and associated active substances, earlier demonstrated to be increased in women with pelvic pain, modulate the miRNA profile. Further investigation into this entity might promote our understanding of the pelvic pain mechanism and development of the therapies for women with chronic pelvic pain. This information is also valuable to assess confounding effect of pelvic pain in further studies of miRNAs in eutopic endometrium.

Over-expression of miR-551a appeared to be consistently unique to the eutopic endometrium from women with endometriosis. MiR-551a promotes tumour cell motility, invasion, and metastasis via suppression of PRL-3 gene in gastric cancer and thus may have a role in ectopic endometrial implantation in the pelvis. Additionally the predicted targets of miR-551a include transcripts that alter TNF-α-induced regulation of IL-6/IL-8 and VEGF translation which has functional relevance for endometriosis.

Finally, our results did not reveal a correlation between eutopic endometrium and the plasma miRNA profile in endometriosis, suggesting that miRNAs in plasma are not passively released from eutopic endometrial tissue. However, circulating miRNAs may modulate cell function in

eutopic endometrium, further supporting an assumption that endometrial alterations are likely related to the disease progression, rather than an initiating pathogenetic event.

8.7 Plasma miRNAs as diagnostic markers for endometriosis

In a prospective diagnostic study on clinically well-described cohort of 68 women, a set of 4 miRNAs (miR-155, miR-574-3p, miR-133a and miR-30c) displayed aberrant expression in plasma in endometriosis and were incorporated into a predictive model in combination with clinical parameters (Chapter 7). The expression of the identified miRNAs was not confounded by any of the demographic or clinical parameters, suggesting their usefulness as potential biomarkers for a broad unselected population. The differential expression of the miRNAs was not influenced by recurrent disease or a previous diagnosis of endometriosis in currently disease-free women, suggesting that the plasma miRNAs reflect only active ongoing disease, and thus may be valuable as indicators of disease recurrence or response to treatment.

MiR-133a and miR-30c were over-expressed only in the severe forms of the disease particularly in women with ovarian endometrioma. It is possible that these miRNAs represent a unique miRNA profile of endometrioma. If this is the case, concurrent miRNA testing may improve our ability to discriminate endometrioma from other types of ovarian tumours after an ultrasound diagnosis of an ovarian mass.

Our diagnostic accuracy estimates suggest that a plasma miRNA-based predictive model is no more accurate than surgical diagnosis. Further development of the miRNA-based diagnostic algorithm with incorporation of clinical data and biochemical parameters suggest a promising role in improvement of the diagnostic potential of this test. This study was the basis for a provisional patent and successful application for commercial funding to enable investigation of a broader range of miRNA-based markers in larger set of samples. In its present form the model proposed by us can be utilised as a triage test, which would improve surgical selection and provide an opportunity to intervene at earlier time. The lower specificity means that some women will undergo unnecessary surgery, but the number of women without endometriosis who have surgery will be lower than without the test.

When this thesis was in the final stages of drafting, 2 studies on diagnosis of circulating miRNAs in endometriosis were published (211-212), demonstrated inconsistency in the obtained results. Discrepancy between studies on miRNA expression has been demonstrated in other

diseases and tissues, including studies on endometrial tissue in endometriosis. Both technical and biological variability of miRNA expression highlights the complexity of miRNA research and indicates that further exploration is required in the field before we are able to make a confident claim for miRNA-based biomarkers.

In summary, the results of the study included in this thesis present potential novel biomarkers for early detection of endometriosis, although larger sample numbers are required to optimise diagnostic algorithms. The strength of this study was in its prospective nature and in the meticulous design of methodology for both laboratory and clinical aspects of the project, i.e. miRNA quantification, biomarker selection and conduct of the diagnostic study.

8.8 Future perspectives

This study provides an initial insight into circulating miRNAs in association with endometriotic disease. The results of the present study open several areas of investigation.

Firstly, an improved understanding of the source, regulation and mechanism of action of circulating miRNAs may reveal new insights into the pathophysiology and systemic manifestations of endometriosis. A better understanding of the mechanisms through which miRNAs are released in the systemic circulation and/or are taken up by recipient cells will help unravel the role of circulating miRNAs and the mechanism of their regulation. Future studies will need to establish whether endometriosis-specific miRNAs in the bloodstream are contained in exosomes or microparticles, whether they can enter cells and, eventually, which cells and whether they have any biological action.

The present study lays the groundwork for future efforts to identify and develop miRNAs as a novel class of blood-based biomarkers for endometriosis. Our results need to be confirmed in larger patient groups in different populations. Subgroup analyses are required to determine if factors like race, geographical area, disease severity or differences in clinical symptomatology impact on circulating miRNA profiles. Further studies are also required to confirm and to characterise the miRNA profile associated with the presence of ovarian endometrioma and to assess the diagnostic value of these miRNAs to differentiate endometrioma from other ovarian tumours in combination with the imaging modalities. It would also be interesting to assess the diagnostic value of the integrated models, in which miRNA profiles are combined with other types of biomarkers, i.e. Ca-125, VEGF, IL6, IL8, TGF-β and TNF-α. Finally, studies

that assess broader miRNA profiling or using different detection platforms may potentially reveal additional markers.

There is considerable need for a better understanding of the pathophysiology of endometriosis and its epiphenomena and better strategies to translate this knowledge into clinically effective outcomes. Identification of circulating biomarkers of endometriosis with the potential to be developed as a non-invasive blood test for endometriosis has considerable clinical relevance and is expected to reduce the complications of endometriosis, to minimise the healthcare costs and to promote research towards development of more efficient therapies.

Chapter 9
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