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KADRI REKKER

The putative role of microRNAs in endometriosis pathogenesis and potential in diagnostics

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Institute of Clinical Medicine, University of Tartu, Estonia

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LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following original articles:

I **Rekker, K**; Saare, M; Roost, AM; Salumets, A; Peters, M. (2013). Circulating microRNA profile throughout the menstrual cycle. PLoS ONE, 8 (11), e81166.10.1371/journal.pone.0081166.

II **Rekker, K**; Saare, M; Roost, AM; Kaart, T; Sõritsa, D; Karro, H; Sõritsa, A; Simón, C; Salumets, A; Peters, M. (2015). Circulating miR-200-family micro-RNAs have altered plasma levels in patients with endometriosis and vary with blood collection time. Fertility and Sterility, 104 (4), 938−946.10.1016/j.fertnstert.2015.06.029.

III **Rekker**, **K**; Saare, M; Eriste, E; Tasa, T; Kukuškina, V; Roost, AM; Anderson, K; Samuel, K; Karro, H; Salumets, A; Peters, M (2017). High-throughput mRNA sequencing of stromal cells from endometriomas and endometrium. Reproduction, 154 (1), 93−100.10.1530/REP-17-0092.

IV **Rekker, K**; Tasa, T; Saare, M; Samuel, K; Kadastik, Ü; Karro, H; Götte, M; Salumets, A; Peters, M (2018). Differentially-expressed miRNAs in ectopic stromal cells contribute to endometriosis development: the plausible role of miR-139-5p and miR-375. International Journal of Molecular Sciences, 19 (12), pii: E3789.10.3390/ijms19123789.

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Author's personal contribution

Paper I: Participation in the study design and data analysis, performing the experiments, writing the manuscript.

Paper II: Participation in the study design and data analysis, performing the experiments, writing the manuscript.

Paper III: Participation in data analysis, writing the manuscript.

Paper IV: Participation in the study design and data analysis, performing the experiments, writing the manuscript.

ABBREVIATIONS

ASRM	–	American Society for Reproductive Medicine
AUC	–	area under the curve
BMI	–	body mass index
ci-miRNA	–	circulating microRNA
Ct	–	cycle threshold
DEG	–	differentially expressed gene
EMT	–	epithelial-to-mesenchymal transition
ESR1	–	estrogen receptor 1
ESR2	–	estrogen receptor 2
FACS	–	fluorescence-activated cell sorting
FC	–	fold change
HOX	–	homeobox
LH	–	luteinizing hormone
MET	–	mesenchymal-to-epithelial transition
miRNA	–	microRNA
miRNome	–	the full spectrum of microRNAs expressed in a sample of interest
mRNA	–	messenger RNA
NA	–	not applicable
nt	–	nucleotide
pre-miRNA	–	precursor microRNA
pri-miRNA	–	primary microRNA
qRT-PCR	–	quantitative real-time PCR
rASRM	–	revised American Society for Reproductive Medicine
RISC	–	RNA-induced silencing complex
ROC	–	receiver operating characteristic
SCNA	–	somatic DNA copy number alterations
SD	–	standard deviation
TF	–	transcription factor
TGFβ	–	transforming growth factor beta
UTR	–	untranslated region

1. INTRODUCTION

Endometriosis is a gynaecological disease that is characterised by the presence of endometrial-like tissue outside the uterus, forming ectopic endometriotic lesions on the surfaces of peritoneal cavity organs. The disease is affecting around 5–10% of women in reproductive age. Despite the extensive research in the field, diagnostic options for endometriosis are still limited and the only way for definite diagnosis is laparoscopic surgery. The symptoms of the disease are mostly nonspecific, including severe pelvic pain and infertility, which are also related to several other gynaecological disorders. Therefore, numerous women without endometriosis but with similar presentation go through diagnostic laparoscopy. To date, discovering reliable non-invasive biomarkers remains the main research priority in endometriosis. In a search of potential biomarkers for endometriosis, different proteomic and transcriptomic approaches have been utilised and, most recently, microRNAs (miRNAs) have emerged as novel candidate markers.

miRNAs are non-coding RNAs around 18–22 nucleotides in length that function as gene expression modulators. Evolutionary conservation of miRNA sequences among studied species imply to their important role in biological processes. Indeed, studies involving differential expression and functional analysis of miRNAs have confirmed their substantial regulatory effect on gene expression in both normal physiological and pathological conditions.

miRNAs are promising non-invasive biomarker candidates for disease diagnostics as they can be found extracellularly in different body fluids, such as blood serum and plasma, urine and saliva, enabling easy access for testing. As circulating miRNAs (ci-miRNAs) in blood are confined inside extracellular vesicles or bound to proteins, they are also remarkably stable to various conditions. However, although ci-miRNAs are highly stable in biological fluids, their levels seem to be influenced by normal physiological variables, such as age and gender, variations in blood cell composition and circadian cycle. These confounding factors must be taken into account if evaluating the potential of miRNAs as disease biomarkers. In women of reproductive age, prior to studying miRNAs as candidate biomarkers, it is also obligatory to know whether the menstrual cycle affects ci-miRNA levels.

In addition to extensive search for non-invasive diagnostic options, unclear molecular mechanisms of endometriosis pathogenesis have puzzled researchers for decades. Accumulating evidence indicates that miRNAs may be new pieces in the endometriosis puzzle, being involved in the development and persistence of the disease. However, the cellular heterogeneity of endometriotic lesion biopsies, consisting of endometrial specific epithelial and stromal cells, mixed with the cells from the affected peritoneal tissue, hinders the detection of true miRNA patterns characteristic to ectopic endometrial cells. Therefore, to gain insight into the pathological processes occurring in endometrial cells in ectopic locations and to uncover the exact mechanisms leading to the formation of

endometriotic lesions, molecular profiling of each cell type should be performed separately.

Coming from the need for proper non-invasive biomarkers and the lack of knowledge about cell-type specific miRNA alterations in endometriosis, the overall aim of the current study was to evaluate the suitability of miRNAs as endometriosis biomarkers and to assess the potential role of endometriotic stromal cell miRNAs in the disease pathogenesis.

2. REVIEW OF LITERATURE

2.1. microRNAs (miRNAs)

2.1.1. miRNA characteristics, biogenesis and function

microRNAs (miRNAs) are small non-coding RNA molecules ~22 nucleotides in length that regulate gene expression post-transcriptionally by destabilising or repressing the translation from their target mRNAs. miRNAs are generally transcribed from the intronic region of genes or non-coding transcripts, less frequently from the exons of protein coding genes (Rodriguez *et al.* 2004). One miRNA is able to regulate more than a hundred genes (Lewis *et al.* 2005) and each mRNA may be the target for multiple miRNAs (Li *et al.* 2010, Huntzinger and Izaurralde 2011). Over 2,600 annotated mature human miRNAs are known to date [miRBase release 22.1, updated in October 2018 (Kozomara *et al.* 2019)], and as each miRNA regulates multiple genes, it is assumed that miRNAs probably adjust the expression of most genes in the human genome (de Rie *et al.* 2017).

miRNAs are transcribed in the nucleus as primary miRNA molecules (pri-miRNA, Figure 1) that often extend to a few hundred nucleotides (nt) in length. Pri-miRNAs can contain multiple units from which several mature miRNAs are formed. The pri-miRNA is further processed to a ~70 nt stem-loop precursor miRNA (pre-miRNA) which is transported to the cytoplasm. Then the loop sequence of the hairpin is cleaved from the pre-miRNA. During strand selection, one of the two strands (–5' or –3' strand) of the duplex is removed and a single stranded mature miRNA is formed. Functional miRNA binds to the Argonaute protein and is transported to the site of action as the RNA-induced silencing complex (RISC). Whether the miRNA incorporated to the complex is derived from –5' or –3' strand depends on the cell type or cellular environment (Meijer *et al.* 2014). Strand selection determines the name of mature miRNA (miR-xx-3p or miR-xx-5p). RISC with mature miRNA binds to the 3'-untranslated region (UTR) of the mRNA via nucleotides in positions 2 to 8 of the miRNA known as the "seed region" and activates further processes resulting in degradation of target mRNA or inhibition of translation. The magnitude of action depends on a variety of factors, such as the affinity of miRNA "seed region" and mRNA molecule, subcellular location of miRNAs as well as the abundancy of miRNAs and target mRNAs (Molotski and Soen 2012, Denzler *et al.* 2016).

miRNA sequences, in particular the "seed region", are often evolutionarily conserved between different species, which implies that they have essential biological functions (Li et al. 2010, de Rie et al. 2017). Indeed, miRNAs coordinate a wide range of processes such as cellular differentiation (Shenoy and Blelloch 2014), reproduction (Hasuwa et al. 2013) and immunity (Mehta and Baltimore 2016). Although miRNAs are not solely responsible for the gene

expression regulation, even slight alterations from normal miRNA levels can lead to pathological states. Aberrant miRNA expression in tissues is associated with numerous human diseases, including gynaecological pathologies (Teague et al. 2010).

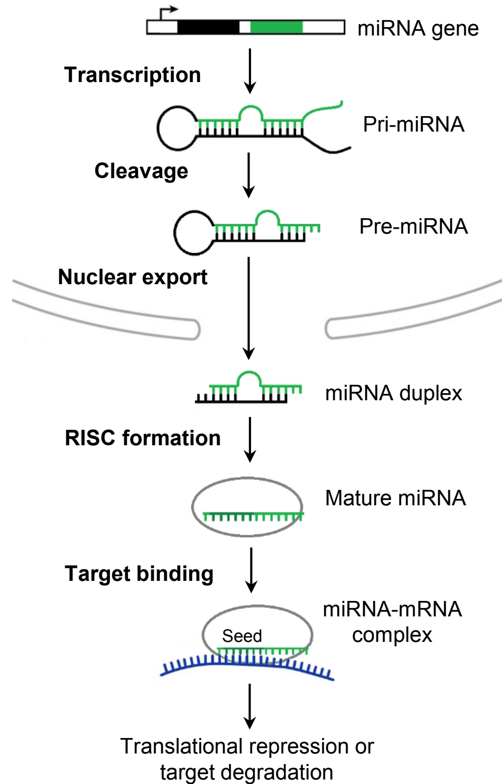

Figure 1. miRNA biogenesis and function.

miRNAs regulate their target mRNA expression prevalently negatively; however, a more complex regulatory networks through a combinatorial action of miRNAs and transcription factors (TFs) are involved in gene expression regulation (Arora et al. 2013). In addition to co-regulation of gene expression, miRNAs and TFs control the expression of each other. The coordinated activity of miRNAs and TFs can occur by different regulatory feedback loops (Krol et al. 2010), where a) miRNA simultaneously represses a TF and a target gene; b) miRNA represses a TF and a target gene, while the TF activates miRNA and the target gene; c) TF simultaneously activates/represses a miRNA and a target gene (Figure 2).

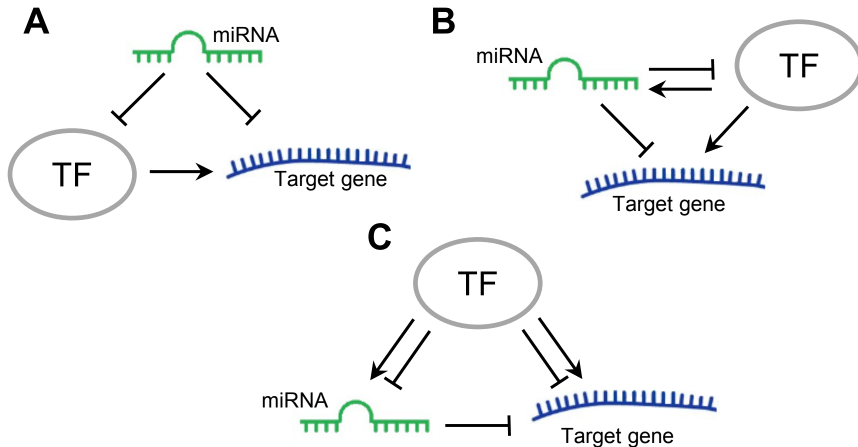

Figure 2. Regulatory networks between miRNAs and transcription factors (TFs) in gene expression regulation.

2.1.2. miRNAs in human cells and tissues

miRNAs have been found in all mammalian tissues and cell types studied so far (Ludwig *et al.* 2016, de Rie *et al.* 2017, McCall *et al.* 2017). Different tissues and cell populations exhibit unique patterns of miRNA expression. Breast tissue samples are one of the most diverse in terms of the number of miRNAs and, in contrast, hair follicles express only a subset of miRNAs (Panwar *et al.* 2017). Some miRNAs are expressed in a majority or all tissue types (Fehlmann *et al.* 2016, Ludwig *et al.* 2016), for instance, let-7 family miRNAs, miR-21, miR-451a and miR-92a are among the most abundant and widespread across different human tissues (Panwar *et al.* 2017, Vitsios *et al.* 2017). Interestingly, miR-21-5p is considered as the top expressed miRNA in humans (Gong *et al.* 2014, Panwar *et al.* 2017).

There are contradicting results whether specific miRNAs are uniquely expressed in particular cell types only (de Rie *et al.* 2017, McCall *et al.* 2017). While some studies have found cell-type specific miRNA expression, others argue that it could be the matter of sequencing depth and miRNAs unique to a certain cell type do not exist (de Rie *et al.* 2017). Nevertheless, it is evident that some miRNAs are highly enriched in particular cell types while other miRNAs are completely absent in specific cell populations (de Rie *et al.* 2017). For example, miR-451a has been described as exclusively expressed in erythrocytes, miR-150 in leukocytes and miR-126 in endothelial cells and platelets (McCall et al. 2011, Kent et al. 2014, McCall et al. 2017).

Tissues are comprised of multiple cell populations and include cell types that constitute the majority of the organ (e.g. hepatocytes in liver), cells from other compartments of the organ (e.g. cells from vascular components) and cells that migrate into in the organ due to stimuli (e.g. macrophages). Tissue biopsies

contain different cell populations in proportions that vary inter-individually or by a location within an organ (Kent et al. 2014). Therefore, tissue-level miRNA expression profile not merely reflects submerged miRNA levels of the cells within a tissue but also the presence and abundance of a certain cell types. For example, miR-451a can be detected in a large variety of tissues including lungs, placenta and thyroid but among cell populations, miR-451a is abundantly expressed only in erythrocytes (McCall et al. 2017). Similarly, miR-126-3p, that is specific to endothelial cells and platelets, is measurable in adrenal gland, skin and lungs (McCall *et al.* 2017). However, as vascular components are a part of almost all tissues, the presence of these miRNAs most probably represent blood cells in the studied sample. Thus, understanding the cellular composition of a sample is essential for interpreting tissue level expression data.

Currently, however, the majority of the studies retrieve information about the overall submerged miRNA expression of the tissue which has clouded our understanding about the true biological role and function of miRNAs (Kent et al. 2014). To overcome the problem, it is important to design the experiments in such way that instead of studying the heterogenous expression profile within a whole tissue, miRNA levels from each compartment of the tissue of interest are determined. Laser capture microdissection or flow cytometry allow to obtain pure populations of cells from heterogeneous tissues and could represent a solution to this issue. Computational deconvolution analysis of global expression is another option to predict fractions of cells within a tissue (Zhao and Simon 2010, Avila Cobos et al. 2018). Applying these approaches would improve the interpretation of tissue miRNA expression data, which is an important step towards the identification of molecular pathways of the disease pathogenesis or cellular targets for drugs.

2.1.3. Circulating miRNAs (ci-miRNAs)

In addition to cells, miRNAs have been found extracellularly in body fluids, such as blood plasma and serum, urine, saliva, tears, peritoneal and seminal fluid (Mitchell *et al.* 2008, Park *et al.* 2009, Hanke *et al.* 2010, Weber *et al.* 2010). Similar to cells and tissues, bodily fluids have distinct miRNA profiles with unique miRNAs specific to a particular sample (El-Mogy *et al.* 2018). Also, there are common miRNAs that have been detected from the majority of biofluids. For example, let-7a-5p, let-7f-5p, miR-191-5p, miR-26a-5p and miR-486-5p are universally present in plasma, serum, saliva and urine samples (El-Mogy *et al.* 2018).

Majority of miRNAs in plasma or serum, most commonly used biofluids for research purposes, originate from blood cells (Pritchard *et al.* 2012). Other tissues contribute to the pool of extracellular miRNAs mainly via transportation by extracellular vesicles (Mitchell *et al.*, 2008). For example, placenta-specific miRNAs in maternal blood plasma are secreted into the circulation via exosomes (Chim *et al.* 2008, Luo *et al.* 2009).

Circulating miRNAs (ci-miRNAs) are recognized as byproducts of cellular injury or death, however, the release of extracellular miRNAs may also be a regulated process (Kosaka *et al.* 2010). miRNAs can be transported into the systemic circulation via extracellular vesicles (Valadi *et al.* 2007, Turchinovich *et al.* 2011) or as ribonucleoprotein complexes (Arroyo *et al.* 2011, Vickers *et al.* 2011). Ci-miRNAs are combined with the recipient cells via endocytosis (Tian et al. 2014) or act through binding receptors at the cell membrane surface (Fabbri *et al.* 2012). Ci-miRNAs are incorporated into distant cells with functional consequences (Valadi *et al.* 2007, Iguchi *et al.* 2010, Kosaka *et al.* 2010); therefore their actions are not limited to the local cellular environment and they rather act as intercellular signalling molecules. Similar to cellular miRNAs, ci-miRNAs induce gene silencing (Iguchi et al., 2010; Kosaka et al., 2010), causing broad range of changes in cell physiology (Cortez *et al.* 2011). For example, exosomal miR-30d is secreted by the endometrial cells in the uterus and is taken up by mouse embryos resulting in altered gene expression (Vilella *et al.* 2015).

2.1.4. Ci-miRNAs as potential biomarkers

Ci-miRNAs possess characteristics that form them into attractive non-invasive biomarker candidates for disease diagnostics. Firstly, they are present in easily accessible body fluids such as blood, urine or saliva. Secondly, contrary to mRNAs, extracellular miRNAs are highly stable despite high concentration of RNases in body fluids (Chen et al. 2008). Thirdly, the expression pattern of ci-miRNAs presents differential levels between control subjects and patients with various pathologies; therefore, ci-miRNAs can be potentially used to identify individuals with cancer as well as infertility and gynaecological issues. With regards to female reproductive system, ci-miRNAs have been proposed as potential biomarkers for preeclampsia, preterm birth, uterine leiomyomata and endometriosis (reviewed in Bjorkman and Taylor 2019).

Among a variety of options, ci-miRNAs have been suggested as biomarkers to diagnose, predict and monitor the progression of diseases as well as response to therapy. As an example of the diagnostic value, increased levels of circulating miR-155, miR-21 and decreased levels of miR-181a-5p have been observed in breast cancer patients in multiple studies (Wang *et al.* 2014, Ferracin *et al.* 2015, Li *et al.* 2016). One of the major obstacles limiting the use of ci-miRNAs in the clinical setting is that the reported miRNAs are largely unspecific. For instance, in addition to breast cancer, the altered level of circulating miR-21 has been associated with colorectal, lung, prostate and endometrial cancers (Zhang *et al.* 2011, Toiyama *et al.* 2013, Yang *et al.* 2014, Gao *et al.* 2016).

Although ci-miRNAs are indicators of the wellbeing status of the organism in pathological conditions, the potential clinical applications of these molecules depend on their reproducibility and stability. Different studies on the same pathology often produce discordant results and potential disease markers are rarely confirmed by other studies. Therefore, ci-miRNAs have not entered to the clinical settings yet.

2.1.5. Factors influencing ci-miRNA levels

2.1.5.1. Biological factors

In addition to pathological processes, several factors are known to influence ci-miRNA levels including biological variables and technical aspects. With regards to biological aspects, demographic and normal physiological variables including age (Sredni *et al.* 2011, Ameling *et al.* 2015), gender (Duttagupta *et al.* 2011, Ameling *et al.* 2015), body mass index (Ameling *et al.* 2015), ethnic origin (Huang et al. 2011) or circadian cycle (Heegaard *et al.* 2016) seem to contribute to ci-miRNA level variations between individuals. Duttagupta *et al.*, showed elevated levels of four ci-miRNAs in women compared to men (Duttagupta *et al.* 2011). Moreover, a wider range in levels of these miRNAs within the studied women was demonstrated compared to a more stable miRNA quantities in men. The source of variation in miRNA levels between men and women could be attributed to differences in blood cell populations as men have elevated erythrocyte counts compared to women (Ameling et al. 2015). Majority of miRNAs in plasma and serum originate from blood cells; therefore, ci-miRNA levels are directly influenced by blood cell counts (Pritchard et al. 2012) and, to some extent, ci-miRNA profiles always reflect the individual blood cell composition at the time of sampling. However, it has been shown that regardless of the blood cell count parameters, ci-miRNA levels are still influenced by sex (Ameling *et al.* 2015) indicating that additional factors must be involved in the variation of plasma miRNA levels among men and women. In reproductive age women, extensive physiological alterations occur every month that are reflected in cyclic changes of miRNA patterns in the endometrium (Kuokkanen *et al.* 2010). Similar to the endometrial tissue, cyclic changes in mammalian ovaries are influenced by miRNAs (McBride *et al.* 2012, Menon *et al.* 2015, Zi *et al.* 2017). Whether the changes in female body during the menstrual cycle have an effect on ci-miRNA levels is not clear.

2.1.5.2. Technical factors

In addition to biological variation, technical aspects including specimen collection and sample handling, miRNA detection methods and subsequent analysis steps have an impact on ci-miRNA quantification, masking the true biological situation.

The origin of variation starts from the initial steps of sample collection and handling. Different additives in blood collection tubes are known to alter miRNA detection levels (McDonald *et al.* 2011) and plasma is known to yield higher quantity of miRNAs compared to serum. Moreover, haemolysis, caused by improper blood collection or preparation, remarkably increases red blood cell derived miRNA levels in plasma (Kirschner et al. 2011, Pritchard et al. 2012). Next, blood centrifugation conditions and subsequent miRNA isolation

steps have a significant influence on ci-miRNAs (Brunet-Vega *et al.* 2015, Li *et al.* 2015, Binderup *et al.* 2016), in particular, guanidine/phenol/chloroform-based methods and commercially available column-based miRNA isolation kits have a variability in yield and ratio of determined miRNA levels. miRNA isolation step is particularly important as potential inhibitors for subsequent reactions (e.g. inhibitors of reverse transcriptase and polymerase enzymes) may co-precipitate with RNA and introduce additional bias among samples (Kim *et al.* 2012).

Small size of miRNAs, similarity in sequence among miRNAs of the same family and large variation in the abundance of different miRNAs (miRNAs with very high and low expression within a sample) challenge miRNA expression profiling in biological samples. Regardless of it, numerous methods have been adapted for miRNA detection, including quantitative real-time PCR (qRT-PCR), microarrays and next generation sequencing. qRT-PCR is considered as the golden standard for miRNA quantification and is one of the most common methods used with samples having low total miRNA levels, such as biofluids. However, there are considerable inter-platform differences when evaluating miRNA expression even with the same technological platform but using reagents from different companies (Git et al. 2010, Mestdagh et al. 2014). Inter-platform variation tends to increase with biofluid samples due to lower amount of total miRNA levels (Chen et al. 2009).

Data normalisation is another major issue when tackling with the effects of experimental variation. The purpose of normalisation is to reduce the technical variability and obtain the true biological results. Unlike in mRNA expression analysis, there are no housekeeping miRNAs that can be universally used as a reference for normalisation, in particular for biofluid samples. The most widely used normalisation approaches for ci-miRNAs employ specific miRNAs or a combination of miRNAs with stable levels among studied sample type. Conflictingly, a widely used miRNA for normalisation – miR-16 – has been reported as differentially expressed in several diseases, including endometriosis (Suryawanshi *et al.* 2013). In addition, small RNAs, external RNA ‘spike-in’ molecules and global mean of all measured miRNAs are applied for normalisation of miRNA levels. Other classes of small RNAs are frequently used as reference markers, however small RNAs like U6 display high inter-individual variance and are not suitable for serum/plasma samples (Qi *et al.* 2012, Benz *et al.* 2013, Nisenblat *et al.* 2019). A global expression mean seems to function efficiently to normalise miRNA levels (Mestdagh et al. 2009); however, this approach can only be adapted for high-throughput analysis.

Inconsistent results between different studies due to technical and experimental setup as well as inter-individual variation create controversy among discovered miRNA findings. Prior to translating the discoveries of ci-miRNAs into the clinical practice, a clear understanding of all aspects that affect ci-miRNA levels is needed in order to create standardised protocols essential in medical settings.

2.2. Endometriosis

2.2.1. Endometriosis epidemiology

Endometriosis is a frequent gynaecological disease, that is characterized by the presence of endometrium-like tissue outside the uterus. Ectopic endometrial tissue can form lesions on ovaries and other peritoneal organs, in rare cases, endometriosis occurs in extraperitoneal locations including the colon, kidney, liver, pancreas, and lungs (Flieder et al. 1998, Sonavane et al. 2011).

Endometriosis-specific symptoms do not exist. Most commonly, women with the disease suffer from pelvic pain that is also related to a number of other conditions (May *et al.* 2010) and around 30–50% of endometriosis patients have fertility problems (Meuleman *et al.* 2009). The prevalence of the disease is estimated around 5–10% in reproductive age women (Hummelshoj *et al.* 2006, de Ziegler *et al.* 2010); however, the exact number of women suffering from endometriosis is difficult to determine as the disease is occasionally asymptomatic and often misdiagnosed.

Three different forms of endometriotic lesions have been characterized: peritoneal lesions, ovarian endometriomas and deep infiltrating lesions. Endometrioma – benign ovarian cyst containing thick fluid formed from degenerated blood – is the most common type of lesions. The morphology, anatomical location and the appearance of lesions refer to uncommon pathogenesis patterns and, therefore, different types of endometriosis are generally recognized as distinct clinical entities (Nisolle and Donnez 1997). The clinical presentation of the disease is often heterogenous and mixed types of endometriotic lesions are frequently present, accompanied by adhesions. According to the most widely used clinical classification system, formulated by American Society for Reproductive Medicine (ASRM), endometriosis is divided into four stages: I – minimal, II – mild, III – moderate and IV – severe (ASRM, 1997). Stage I and II endometriosis comprise of superficial endometriotic lesions with or without adhesions; stage III and IV include an endometrioma or endometrioma in combination with superficial or deep lesions, in addition to minor to severe adhesions. Interestingly, no correlation between the symptoms (including severity of pain and fertility outcome) and the staging system exists (Vercellini *et al.* 2007).

2.2.2. Ectopic tissue formation

The origin of endometrial tissue in ectopic locations is mainly accepted to be via retrograde menstruation where menstrual blood, containing viable endometrial cells, flows back through the fallopian tubes into the pelvic cavity (Sampson 1927). The shed endometrial cells attach to the peritoneum, proliferate and differentiate, forming lesions. However, as retrograde menstruation is a common process (Halme *et al.* 1984) other factors must be involved in endo-

metriotic lesion formation. The establishment of lesions requires a favourable endocrine and metabolic environment in ectopic sites that includes altered immune and inflammatory responses (Olovsson 2011, Ahn *et al.* 2015). In the peritoneal cavity, the changes that promote cell survival, proliferation, angiogenesis and altered apoptosis have been proposed to induce the formation of endometriotic lesions. For example, altered level of cytokines in the peritoneal fluid, that are secreted by lymphocytes in response to inflammatory stimuli, have been reported in endometriosis patients (Kyama *et al.* 2009).

As a highly heterogeneous disease, endometriosis cannot solely be explained by retrograde menstruation theory and the pathophysiology is more likely multifactorial (Nisolle and Donnez 1997), involving an interplay between various pathology scenarios combined with other factors. It has been proposed that the ovarian endometrioma formation arises from the accumulation of menstrual debris, derived from the bleeding of superficial endometriotic lesions, resulting in invagination of ovarian cortex (Brosens et al. 1994, Vercellini et al. 1998) or due to the metaplasia of the invaginated ovarian tissue (Nisolle and Donnez 1997, Matsuura et al. 1999). Endometriotic lesions in distant extraperitoneal locations can be explained by the migration of endometrial cells through lymphatic system (Donnez et al. 2002, Tempfer et al. 2011). Recent studies also suggest that endometriosis may arise from dislocated or aberrant stem cells (Gargett *et al.* 2014). Moreover, genetic factors may play a role whether an individual develops endometriosis in a lifetime as close relatives of endometriosis patients are at higher risk of developing the disease themselves (Treloar *et al.* 1999). Genetic background is further proven by the most recent genome wide association study, implicating the role of genes involved in sex steroid hormone pathways, including estrogen receptor 1 (*ESR1*) gene, in endometriosis pathogenesis (Sapkota *et al.* 2017). Nevertheless, the pathogenesis of endometriosis, in particular on the molecular level, is still poorly understood and controversial.

2.2.3. Endometriosis diagnosis

Invasive laparoscopic surgery is the main diagnostic and treatment option for endometriosis. However, because of non-specific symptoms of the disease and the lack of clinically relevant non-invasive testing options, there is a long delay between the onset of symptoms and the diagnosis of endometriosis, averaging around 7 years (Nnoaham *et al.* 2011). Although several non-invasive techniques, including ultrasonography and magnetic resonance imaging, can aid the diagnosis by identifying the presence of ovarian endometriomas or deep infiltrating endometriosis (Hsu *et al.* 2010), detecting peritoneal lesions still remains challenging. Furthermore, owing to the nonspecific symptoms of the disease, numerous women without endometriosis but similar presentation go through a surgery.

Finding diagnostic tools with sufficient diagnostic power is highlighted as one of the research priorities for endometriosis (Rogers *et al.* 2017). A number of studies have described potential non- or semi-invasive biomarker candidates for endometriosis from menstrual or peripheral blood, urine, and endometrial biopsies (Liu *et al.* 2015, Gupta *et al.* 2016, Nisenblat *et al.* 2016). Glycoproteins, cytokines, immune cells and immunological markers, molecules related to cell adhesion, invasion and angiogenesis, transcriptomic, epigenetic and miRNA markers have been described as differentially expressed between patients with and without endometriosis. The most widely studied marker for endometriosis is CA-125 (Fassbender *et al.* 2015), however, elevated levels of this molecule in blood are not specific to endometriosis and CA-125 is more widely accepted as a cancer biomarker. Despite the research effort made in the field, none of the proposed biomarkers have demonstrated reliable diagnostic value in clinical settings (Nisenblat *et al.* 2016).

2.2.4. Eutopic and ectopic endometrium

2.2.4.1. Histological characteristics

Endometrium is a dynamic tissue lining the uterus, which undergoes cyclic growth and regression with each menstrual cycle. Histologically, endometrium primarily consists of two cell types – epithelial cells (forming luminal surface epithelium and glands) and stromal cells (Figure 3A). In addition, endothelial cells and leukocytes are present. As a highly regenerative tissue, the endometrium also contains various cell populations with stem cell characteristics (Chan *et al.* 2004, Patel *et al.* 2008, Maruyama *et al.* 2010).

Endometriotic lesions are composed of similar structural elements as the endometrium (Figure 3B). The main components are glandular epithelial cells surrounded by stromal cells embedded into the surrounding non-endometriotic cells. The proportion of epithelial and stromal cells in the ectopic tissues varies considerably. In addition, inflammatory, endothelial, smooth muscle cells, hemosiderin deposits and fibrotic tissue are often observed via histological assessment of biopsied ectopic tissues (Jansen and Russell 1986, Moen and Halvorsen 1992, Matsuzaki *et al.* 1998, Anaf *et al.* 2000, Anaf *et al.* 2006). Similar to the endometrium, the endometrial-like lesions outside the uterine cavity go through cyclic proliferation and breakdown. This internal bleeding results in inflammation causing adhesions during repair processes.

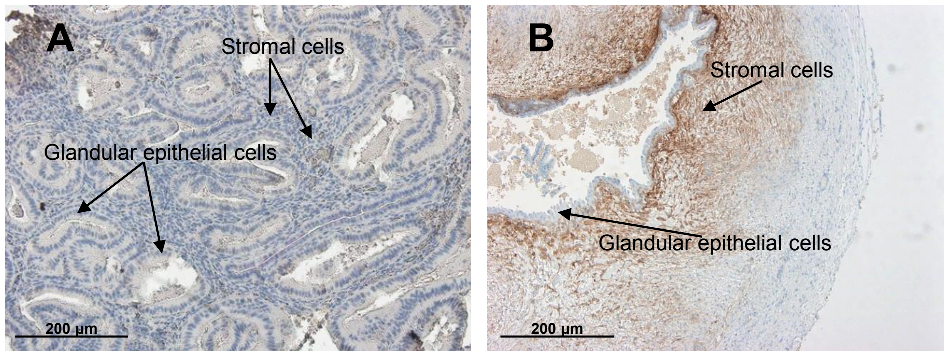

Figure 3. Histological image of endometrial (**A**) and endometrioma (**B**) tissue section from endometriosis patient. 10 × magnification, 10 µm section stained with hematoxylin (**A**) or hematoxylin and CD10+ (**B**). CD10+ stromal cells in endometrioma tissue section are visualised in brown (**B**). Image courtesy of Merli Saare from the Institute of Clinical Medicine, University of Tartu.

2.2.4.2. Molecular characteristics

On a molecular level, eutopic and ectopic endometrium differ considerably and alterations in (epi)genetic, transcriptomic and proteomic signatures have been described. Regarding genetic alterations, somatic mutations, including known cancer driver mutations in *ARID1A, PIK3CA, KRAS* and *PPP2R1A* genes have been shown in deep infiltrating endometriotic lesions (Anglesio *et al.* 2017). Interestingly, these mutations were detected only in the epithelium and not in stromal cells. Still, endometriosis is considered a benign disease and the authors speculated that these mutations are characteristic to endometriosis pathogenesis and are not related to endometriosis-related cancer as the presence of driver mutations alone is not sufficient to drive the transformation of endometriosis to malignancy. There is no clear consensus whether the somatic DNA copy number alterations (SCNAs) in endometriotic lesions exist but we have previously shown that endometriosis-specific SCNAs are not common, indicating that molecular mechanisms other than chromosomal rearrangements are behind endometriosis pathogenesis (Saare *et al.* 2012).

Epigenetic modifications, including DNA methylation and histone modification have been thoroughly studied in the recent years (reviewed in Borghese *et al.* 2017). As an example, the DNA in endometriotic stromal cells is globally hypomethylated compared to eutopic cells due to the downregulation of DNA methyltransferase 1 (Hsiao *et al.* 2015). Also, estrogen receptor 2 (*ESR2*) and GATA family transcription factors are among genes with strong methylation pattern alterations in ectopic endometrium, pointing to the malfunctional hormonal pathway regulation in lesions (Borghese *et al.* 2010, Dyson *et al.* 2014).

There are major differences in transcriptomic patterns between eutopic and ectopic tissues and thousands of dysregulated genes in endometriotic cells have

been reported. For instance, in ectopic endometrium, homeobox (HOX) genes, including *HOXA10* and *HOXA11* are downregulated (Borghese *et al.* 2008, Szczepanska *et al.* 2012). HOX genes are highly conserved transcription factors which main function is to specify the head-to-tail axis in the developing embryo (Pearson *et al.* 2005). In relation to endometriosis, it has been speculated that aberrant HOXA10 expression contributes to disease pathogenesis by inducing progesterone resistance (Browne and Taylor 2006). In addition, genes encoding complement components (*C1R*, *C3*, and *C7*) are upregulated in ectopic endometrium (Tao *et al.* 1997, Eyster *et al.* 2002). These genes are involved in the activation of complement cascade pathways and thus play a role in inflammatory reactions. The dysregulation of complement genes may contribute to the immunological dysfunction associated with the disease (Eyster et al. 2002). Other dysregulated genes in ectopic tissues are involved in processes such as cell adhesion (integrins) (Sundqvist *et al.* 2012), proliferation (*PDGFR*) (Matsuzaki *et al.* 2004), invasion (*MMP*s) (Cox *et al.* 2001, Di Carlo *et al.* 2009) and angiogenesis (*VEGFA*) (Di Carlo *et al.* 2009).

A few proteomic analyses that have been conducted with ectopic endometrial tissue or cells demonstrate altered protein levels in lesions (Kyama *et al.* 2006, Chehna-Patel *et al.* 2010, Marianowski *et al.* 2013, Vehmas *et al.* 2014, Kasvandik *et al.* 2016). As an example, dysregulated levels of transforming growth factor beta (TGFβ) in ectopic lesions have been shown (Vehmas *et al.* 2014, Kasvandik *et al.* 2016). Interestingly, TGFβ is associated with endometriotic lesion growth and increased levels of TGFβ have also been reported in the peritoneal fluid, serum and the peritoneum of women with endometriosis (Young *et al.* 2017). Based on the proteomic data, Kasvandik *et al*. proposed a Warburg effect-like reprogramming of endometriotic stromal cells with increased glycolysis and reduced oxidative respiration leading to tissue growth (Kasvandik *et al.* 2016).

2.3. Endometriosis and miRNAs

2.3.1. miRNAs in endometriotic tissue

Like proteomic, transcriptomic and genomic alterations, dysregulated miRNAs have been identified in the eutopic (Burney *et al.* 2009, Aghajanova and Giudice 2011, Ramon *et al.* 2011, Braza-Boils *et al.* 2014, Zheng *et al.* 2014) and ectopic endometrium (Table 1) of endometriosis patients. Studies focusing on the comparison of miRNA expression profiles in the endometria of women with and without endometriosis have highlighted the downregulation of miR-9 and miR-34 families (Burney *et al.* 2009) or upregulation of miR-21 in endometriosis patients (Aghajanova and Giudice 2011), suggesting their role in the regulation of progesterone resistance, enhanced proliferation and survival of endometrial cells. It is evident, however, that the eutopic endometrial miRNome is highly influenced by the dynamic changes during the menstrual cycle

(Petracco *et al.* 2011, Vilella *et al.* 2015, Sigurgeirsson *et al.* 2017, Rekker *et al.* 2018). As an example, the endometrial miR-135a and miR-135b levels fluctuate during the menstrual cycle being lowest at the time of ovulation (Petracco *et al.* 2011). Nevertheless, some of the abovementioned studies have failed to take this variance into account and analysed samples from different menstrual cycle phases together, questioning the relevance of discovered findings in endometriosis pathogenesis.

In addition, differences between the eutopic and ectopic endometrial miRNA profiles have been explored. High-throughput approach has been applied to eight of these studies (Table 1) where, mostly, miRNA expression from whole endometriotic lesion biopsies have been compared to eutopic endometrium. However, each study has identified a different subset of miRNAs with limited concordance between the experiments, likely reflecting the heterogeneity in study design including the type of studied endometriotic lesions. Recently, miRNA expression characteristic to each lesion type including endometriomas, peritoneal and deep infiltrating lesions have been shown (Haikalis et al. 2018). The most consistently identified differentially expressed miRNA in endometriosis studies is miR-200b, suggesting that distinct miRNA profiles do exist between ectopic and eutopic tissue. miR-200b has been reported down-regulated in endometriomas (Filigheddu et al. 2010, Hawkins et al. 2011, Braza-Boils et al. 2014) and peritoneal endometriotic lesions (Ohlsson Teague et al. 2009, Shi et al. 2014, Yang et al. 2016).

2.3.2. miRNA-mRNA interactions in endometriosis

Gene expression regulation is a complex process involving an interplay between several participants including miRNAs. The knowledge about the global miRNA-mRNA interactions in endometrial tissues and ectopic lesions is scarce. Zhao *et al*. studied miRNA and gene expression from paired whole tissue biopsies of ectopic and eutopic endometria using high-throughput sequencing (Zhao *et al.* 2018). The authors used bioinformatical approaches and identified miRNA-TF-gene network consisting of 22 miRNAs, 12 TFs and 430 corresponding genes, whereas FOX family TFs, miR-182-5p, miR-106a-5p, *CEBPA* and *SOX9* were among the most important key regulators in ectopic tissues. This analysis also highlighted PI3K-Akt signalling pathway in relation to the disease. The PI3K-Akt pathway was suggested as a possible target for endometriosis treatment as it regulates various cellular functions involved in endometriosis pathogenesis, such as cell growth, differentiation, transformation and survival.

This study also emphasised miR-200 family members as important regulators in endometriosis pathogenesis (Zhao *et al.* 2018). miR-200 family derives from two clusters – miR-200a/miR-200b/miR-429 transcribed from chromosome 1 and miR-200c/miR-141 from chromosome 12 (www.miRBase.org). Among other functions, miR-200 family (in particular miR-200b) regulates TFs *ZEB1* and *ZEB2* that are fundamental factors in epithelial-to-mesenchymal transition (EMT)

and mesenchymal-to-epithelial transition (MET). These processes have frequently been associated with endometriosis pathogenesis (Park *et al.* 2008, Matsuzaki and Darcha 2012). During the EMT, epithelial cells acquire mesenchymal phenotype leading to increased cell invasion and migration (Park et al. 2008). At the molecular level, the downregulation of miR-200b in endometriotic lesions (Ohlsson Teague et al. 2009, Filigheddu et al. 2010, Hawkins et al. 2011, Braza-Boils et al. 2014, Shi et al. 2014, Yang et al. 2016) contributes to these changes by targeting *ZEB1* and *ZEB2* genes which leads to a loss of E-cadherin in endometriotic cells (Eggers et al. 2016).

The role of miR-200 family in endometriosis-related mechanisms has also been emphasised by another high-throughput study, where mRNA and miRNA expression were simultaneously investigated from pure fractions of uncultured eutopic endometrial epithelial and stromal cells of endometriosis patients compared to control women (Logan *et al.* 2018). miR-200b was the most significantly upregulated miRNA in endometrial stromal cells in patients with endometriosis compared to control women. The regulatory actions between *ZEB1* and miR-200b were confirmed by functional analyses and the authors speculated that the refluxed endometrial stromal cells with aberrant miRNA and gene expression, where miR-200b is upregulated and *ZEB1* is downregulated, transform into epithelial cells by MET, and are more prone to form ectopic lesions.

Although an integrated miRNA-mRNA analysis using high-throughput approaches has been conducted from whole endometriotic lesions (Zhao *et al.* 2018) and from eutopic endometrial stromal and epithelial cells from women with and without endometriosis (Logan *et al.* 2018), there are no studies revealing miRNA-mRNA expression patterns from distinct cell types in ectopic tissues. More detailed insight is needed about the regulatory networks in endometriotic cells to understand endometriosis at the molecular level.

Table 1. High-throughput miRNA expression studies in eutopic and ectopic endometrial tissue.

Study	**miRNA detection method**	**Studied samples**	**Menstrual cycle phase**	**Dysregulated miRNAs**
(Ohlsson Teague *et al.* 2009)	Custom microarray with 377 miRNAs	Peritoneal, ectopic (n=7), eutopic (n=7)	Proliferative/ secretory	Upregulated: miR-145, 143, 99a, 99b, 126, 100, 125b, 150, 125a, 223, 194, 365, 29c and 1 Downregulated: miR-200a, 141, 200b, 142-3p, 424, 34c, 20a and 196b
(Filigheddu *et al.* 2010)	Custom microfluidic chip with 475 miRNAs	Ovarian, ectopic (n=16), eutopic (n=16)	Early proliferative	Upregulated: miR-1, 100, 101, 126, 130a, 143, 145, 148a, 150, 186, 199a, 202, 221, 28, 299-5p, 29b, 29c, 30e-3p, 30e-5p, 34a, 365, 368, 376a, 379, 411, 493-5p and 99a Downregulated: miR-106a, 106b, 130b, 132, 17-5p, 182, 183, 196b, 200a, 200b, 200c, 20a, 25, 375, 425-5p, 486, 503, 638, 663, 671, 768-3p, 768-5p and 93
(Hawkins *et al.* 2011)	Small RNA sequencing	Ovarian, ectopic (n=10), control eutopic (n=11)	Proliferative/ secretory/ interval/ unknown	Upregulated: miR-202, 193a-3p, 29c, 708, 509-3-5p, 574-3p, 193a-5p, 485-3p, 100, and 720; Downregulated: miR-504, 141, 429, 203, 10a, 200b, 873, 200c, 200a, 449b, 375, and 34c-5p
(Abe *et al.* 2013)	Agilent human miRNA microarray for 955 miRNAs	Ovarian, ectopic (n=26); control eutopic (n=18)	Proliferative	Upregulated: miR-210, 100, 132*, 181a Downregulated: miR-199b-5p, 503, 424, 196b, 199a-3p, 214, 29b, 455-3p
(Shi *et al.* 2014)	miRCURY LNA™ Array for 1,700 miRNAs	Not specified	Proliferative	Upregulated: miR-1915, 637, 518e*, 519a*, 519b-5p, 519c-5p, 522*, 523*, 574-5p, 615-3p, 1909, 224*, 133b, 622, 628-3p, 1470, 1469, 520d-5p, 551b*, 361-3p, 941, 202, 663b, 381, 412 Downregulated: miR-203, 425, 183, 92a, 196b, 363*, let-7i, 200b, 215, 362-3p, 342-3p, 200c, 93, 24-1*, 25, 106b*

Study	**miRNA detection method**	**Studied samples**	**Menstrual cycle phase**	**Dysregulated miRNAs**
(Braza-Boils *et al*. 2014)	GeneChip miRNA 2.0 Affymetrix array for 1,105 miRNAs	Ovarian, ectopic (n=51); peritoneal, ectopic (n=18), rectovaginal, ectopic (n=20); eutopic (n=51); control eutopic (n=32)	Proliferative/ secretory	79 up- and 77 downregulated miRNAs
(Yang *et al*. 2016)	Affymetrix GeneChip miRNA array	Ovarian, ectopic (n=32); control eutopic (n=19)	Not specified	Upregulated: miR-16-5p, 106b-5p, and 145-5p Downregulated: miR-200b, 15a-5p, 19b-1-5p, 146a-5p, and 200c
(Zhao *et al*. 2018)	Small RNA sequencing	Ovarian, ectopic (n=30), eutopic (n=30)	Secretory	41 up- and 66 downregulated miRNAs

Asterisk (*) denominates miRNAs with less expressed strand according to previous nomenclature system.

2.3.3. Ci-miRNAs in endometriosis

Endometriosis still lacks reliable non-invasive diagnostic test, which is one of the major hurdles for timely disease diagnosis. Although considerable efforts have been made to identify endometriosis biomarkers, none with acceptable diagnostic value have been identified. In recent years, ci-miRNAs have been intensively evaluated as candidate markers for several diseases, including endometriosis.

To date, seven studies have examined ci-miRNA profiles from women with and without endometriosis using high-throughput assays (Table 2). Varieties in experimental design of these studies include sample type (plasma or serum), miRNA detection platform, selection of control group (laparoscopically confirmed endometriosis-free women or healthy volunteers). Other technical variations among studies are differences in RNA isolation and normalisation methods (reviewed in Agrawal *et al.* 2018). The sample size in published studies is generally small or moderate ranging from 20 to 80 cases and controls. Only one study has replicated the findings in an independent cohort of women with and without endometriosis in addition to patients used for the discovery and validation phase (Nisenblat et al. 2019).

All seven studies describe significantly different ci-miRNA levels between control subjects and patients with endometriosis with varying levels of diagnostic accuracy. However, each study reports a different set of ci-miRNAs as endometriosis biomarker candidates and the consistency between the results is remarkably small (Agrawal *et al.* 2018). Several miRNAs have been displayed with accuracy high enough to suggest utility for diagnostic purposes. Most intriguingly, Cosar *et al*. presented a combination of three miRNAs – miR-125b-5p, miR-451a and miR-3613-5p – that was able to distinguish endometriosis patients from control subjects with 100% sensitivity and specificity (Cosar *et al.* 2016). Despite this fact, none of the ci-miRNAs have been applied to clinical practise for endometriosis.

2.4. Summary of literature review

Endometriosis has been studied extensively and the information about why and how the disease develops is growing rapidly. However, the molecular pathogenesis mechanisms of the condition are still unclear and no proper markers for endometriosis diagnostics have been found that could be used prior the surgery in women with a suspicion of the disease.

Most lately, miRNAs have been shown to be involved in the regulation of various biological processes and could represent a solution to the unclear mechanisms of the disease pathogenesis and lack of markers in endometriosis diagnostics. However, a more thorough research is needed about the cell type specific miRNA expression patterns to detect the true biological processes behind the disease pathogenesis. This could also lead to the discovery of specific biomarkers and therapeutic targets for endometriosis.

Table 2. High-throughput studies on ci-miRNAs in endometriosis.

Study	Method	Sample type	Study participants	Reference for qRT-PCR normalisation	Validated dysregulated miRNAs
(Jia *et al.* 2013)	Agilent microarray with 1,205 miRNAs	Plasma	Stage III–IV endometriosis patients (n=23), surgically confirmed endometriosis-free patients (n=23)	miR-16	Downregulated: miR-17-5p, 20a, 22
(Wang *et al.* 2013)	TaqMan miRNA array for 765 miRNAs	Serum	Endometriosis patients (n=60); surgically confirmed endometriosis-free patients (n=25)	U6	Upregulated: miR-199a, 122 Downregulated: miR-9*, 145*, 141*, 542-3p
(Suryawanshi *et al.* 2013)	Human miRNome Profiler kit (System Biosciences) for 1,113 miRNAs	Plasma	Endometriosis patients (n=33) and healthy women (n=20)	miR-132	Upregulated: miR-16, 191, 195
(Hsu *et al.* 2014)	SeraMir microarray for 380 miRNAs	Serum	Endometriosis patients (n=40) and surgically confirmed endometriosis-free patients (n=25)	18S RNA	Downregulated: miR-199a-5p
(Wang *et al.* 2016)	Solexa sequencing	Serum	Endometriosis patients (n=30) and surgically confirmed endometriosis-free patients (n=20)	cel-miR-39	Upregulated: miR-424-3p, 185-5p Downregulated: miR-30c-5p, 127-3p, 99b-5p, 15b-5p, 20a-5p
(Cosar *et al.* 2016)	Affymetrix miRNA 4.0 Arrays for 1,205 miRNAs	Serum	Endometriosis patients (n=24) and surgically confirmed endometriosis-free patients (n=24)	U6	Upregulated: miR-125b-5p, 150-5p, 342-3p, 145-5p, 143-3p, 500a-3p, 451a, 18a-5p Downregulated: miR-6755-3p, 3613-5p
(Nisenblat *et al.* 2019)	TaqMan Low Density Human miRNA arrays for 667 miRNAs	Plasma	Endometriosis patients (n=157), surgically confirmed endometriosis-free patients (n=76), healthy women (n=16)	miR-30b	Downregulated: miR-155, 574-3p, 139-3p

Asterisk (*) denominates miRNAs with less expressed strand according to previous nomenclature system.

3. AIMS OF THE STUDY

The general aim of the study was to assess the role of miRNAs in endometriosis pathogenesis and their suitability as non-invasive biomarkers. The specific aims of the study were accordingly:

a) to evaluate the plasma ci-miRNA profile in healthy women throughout the menstrual cycle;
b) to determine the levels of plasma miR-200a, miR-200b and miR-141 in endometriosis patients and evaluate their suitability as diagnostic biomarkers for the disease;
c) to describe miRNA expression pattern in ectopic endometrial stromal cells and, together with transcriptomic data, determine their potential underlying biological mechanisms related to endometriosis pathogenesis.

4. MATERIALS AND METHODS

4.1. Ethics statement

The study was approved by the Research Ethics Committee of the University of Tartu, Estonia (approval numbers 191T-18, 247M-20, 265M-13, 276M-13 and 221/M-31). Written informed consent was signed by all women who entered the study.

4.2. Overview of the study design

This thesis is based on four published studies. General characteristics of all study participants are presented in Table 3. "Healthy participants" comprised of the subjects who defined themselves as healthy and were recruited for Studies I and II. None of the healthy volunteers suffered from gynaecological, inflammatory or chronic diseases at the time of participation. No previous history of endometriosis or autoimmune disorders was reported within these individuals.

Patients undergoing laparoscopic surgery at Tartu University Hospital's Women's Clinic (Estonia) or Elite Clinic (Estonia) with a suspicion of endometriosis and/or complaints of severe dysmenorrhoea or infertility were enrolled in the Studies II, III and IV. Presence of endometriosis in a study group "patients with endometriosis" was confirmed by surgical and histological findings. The stage of endometriosis was assessed according to the revised ASRM classification system (ASRM, 1997) and was classified as minimal–mild (stage I–II) or moderate–severe (stage III–IV). Study group defined as "patients without endometriosis" was confirmed as endometriosis-free by laparoscopy.

Two out of eight healthy volunteers from additional study group (Study II) were using hormonal contraceptive medications at the time of participation. None of the other women had used hormonal medications for at least three months prior the study.

An overview of the study design is depicted in Figure 4 and briefly described below.

In the first study, ci-miRNA profiling was performed from blood plasma samples collected from nine heathy women at four different time points within a menstrual cycle using Exiqon miRCURY LNA microRNA Human panel I assays (Study I). Also, miRNAs from whole blood (buffy coat) were determined from pooled RNA samples of six participants (out of the same nine women used for plasma miRNA profiling) at four menstrual cycle time points.

Secondly, circulating miR-200-family miRNA levels were compared between 30 healthy participants, 35 patients without endometriosis and 61 patients with endometriosis using qRT-PCR. In addition, eight volunteers were recruited to validate the circadian fluctuations of studied miRNAs (Study II).

Thirdly, eutopic and ectopic endometrial stroma mRNA (Study III, n=4+4) and miRNA (Study IV, n=4+4) patterns were determined from uncultured stromal cells isolated from paired samples of endometriomas and eutopic endometrium using next-generation sequencing. For validation, additional samples from five eutopic and six ectopic tissues from endometriosis patients were used in mRNA experiments (in total n=9+10 samples) and two additional paired samples (in total n=6+6) in miRNA experiments. In addition, cultured cells isolated from eutopic and ectopic tissues (n=6+6) were used in validation (Study IV). The samples were collected from different patients for Studies III and IV.

Data analysis was performed in collaboration with Tanel Kaart, PhD (Study II) from the Estonian University of Life Sciences, Viktorija Kukuškina, MSc (Study III) and Tõnis Tasa, MSc (Studies III, IV) from the Institute of Genomics at the University of Tartu.

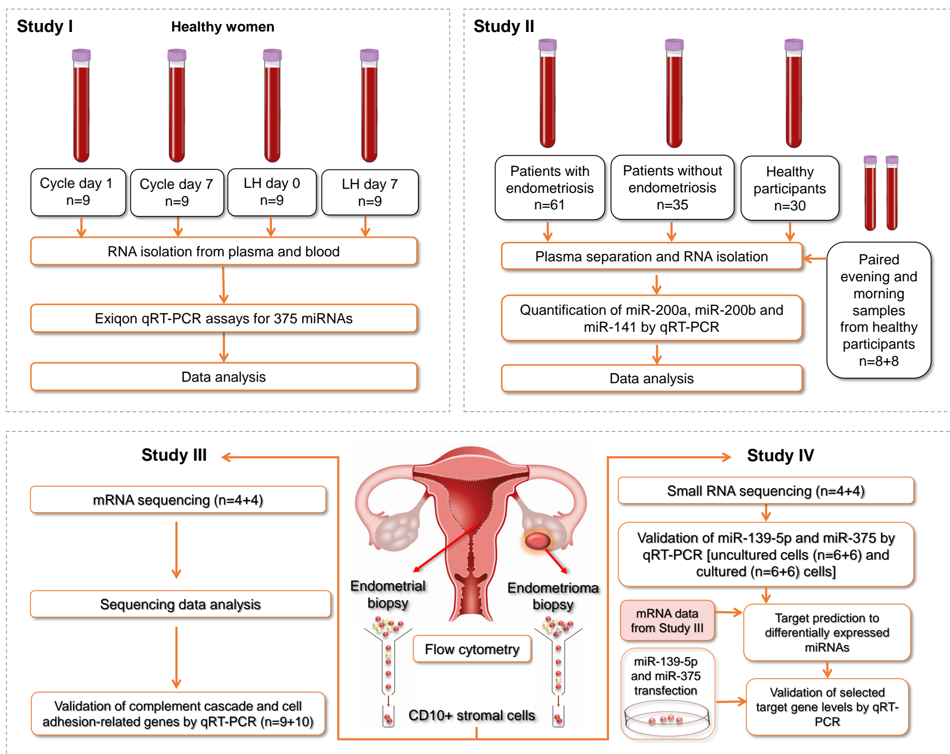

Figure 4. A graphical presentation of the study design. qRT-PCR – quantitative real-time PCR.

Table 3. Clinical characteristics of the study participants.

	Study I	Study II			Study III	Study IV
Study group	Healthy participants	Healthy participants	Patients without endometriosis	Patients with endometriosis	Patients with endometriosis	Patients with endometriosis
Number of participants	9	30+8*	35	61	10	12
Studied sample type	Blood plasma/whole blood (buffy coat)	Blood plasma			Endometrial eutopic and ectopic stromal cells	Endometrial eutopic and ectopic stromal cells
Menstrual/ endometrial cycle time-point	Cycle day 1; cycle day 7; LH+0; LH+7; n=9	Follicular n=19; luteal n=11; not known n=8	Follicular n=12; luteal n=22; not known n=1	Follicular n=24; luteal n=36; not known n=1	Proliferative	Proliferative
Age (year ±SD)	26±5.5	31±8.2	31±5.5	32±5.0	33±5.1	32.0±6.6
BMI (kg/m^2±SD)	22±3.6	23±3.4	24±4.9	23±3.5	21±3.5	22.4±2.4
Endometriosis stage (rASRM)	NA	NA	NA	I–II stage n=33; III–IV stage n=28	I–II stage n=1; III–IV stage n=9	III–IV stage n=12

Data are expressed as mean±SD. BMI – body mass index; LH – luteinizing hormone; rASRM – revised American Society for Reproductive Medicine; NA – not applicable; SD – standard deviation; follicular phase – menstrual cycle day 1–14, luteal phase – menstrual cycle day 15–28. *Healthy participants were recruited as a control group (n=30) and additional group (n=8) to validate the circadian fluctuation of studied miRNAs.

4.3. Sample collection and processing

In total, 151 peripheral blood samples (Study I and II) were collected into EDTA tubes and were processed within an hour after collection. Samples in Study I were taken at four time points within one menstrual cycle – the first day of the menstruation (cycle day 1); the seventh day from the beginning of the menstruation (cycle day 7); the time of the luteinizing hormone (LH) surge (LH+0) and seven days after the LH surge (LH+7). The time of the LH surge was assessed with urine-based commercial LH test. From patients attending the hospital for scheduled surgery, blood samples were obtained one day before the surgery (in the evening) or in the morning prior the anaesthesia for the laparoscopy. From an additional study group of healthy volunteers (Study II), blood samples were collected in the evening/afternoon and in the consecutive morning to determine the possible circadian fluctuation of studied plasma miRNA levels.

Plasma was isolated by two centrifugations at 1,600*g* for 10 minutes and at 16,000*g* for 10 minutes at 4 °C. After the first centrifugation, buffy coat and plasma fractions were collected into separate tubes. Plasma (Study I and II) and buffy coat samples (Study II) were stored at –80 °C with three volumes of TRIzol LS Reagent (Invitrogen, Life Technologies, USA; Study I) or without any additives (Study II) until further use. RNA isolation from was conducted using miRNeasy Mini kit (Qiagen, Germany).

Endometrial and endometrioma tissue samples were collected during the laparoscopy for suspected endometriosis or infertility (Study III and IV). Endometrial samples were obtained using a Pipelle endometrial suction catheter (Laboratoire CCD, France). All collected tissue samples were immediately placed into the cryopreservation medium then into cell freezing containers and deposited in a –80 °C freezer overnight. The frozen biopsies were stored in liquid nitrogen until further use.

4.4. miRNA profiling from blood samples and data analysis (Studies I and II)

miRCURY LNA microRNA Human panel I (Exiqon, Denmark) qRT-PCR assays were used to determine the expression profile of 375 circulating plasma miRNAs at four time points (cycle day 1 and 7, LH+0 and LH+7) within one menstrual cycle of healthy participants (Study I). In addition, pooled whole blood (buffy coat) RNA samples (six samples within each pool) were used for miRNA profiling from four menstrual cycle time points using the same platform. Circulating plasma miR-200 family miRNA levels (miR-200a, miR-200b and miR-141) from Study II were analysed with qRT-PCR using TaqMan microRNA assays (Applied Biosystems, USA).

Relative expression values for ci-miRNA levels for Studies I and II were calculated from average cycle threshold (Ct) values using formula: $\Delta Ct = Ct_{miRNA} - Ct_{Ref}$, where ΔCt is a relative miRNA expression value, Ct_{miRNA} is an average Ct value of miRNA of interest and Ct_{Ref} is an average Ct value of reference molecules. qRT-PCR data set in Study I was normalised by global mean strategy (Mestdagh *et al.* 2009) using GenEx software 2.0 (MultiD Analyses, Sweden). Combined average levels of miR-30e-5p and miR-99a-5p were used as references for data normalisation in Study II. Fold change values (FC) were calculated by formula $FC=2^{-\Delta\Delta Ct}$ (Livak and Schmittgen 2001).

Relative miRNA expression level values (ΔCt) were compared between the menstrual cycle time points with one-factor ANOVA (Study I). Differences in miRNA levels between blood and plasma samples were determined by two-tailed unpaired t-test. P-values were adjusted for multiple testing according to the Bonferroni correction. A two-factor ANOVA (without replication) was performed on the number of detected plasma miRNAs to assess the differences between the menstrual cycle time points and studied individuals. In this analysis, p-value ≤ 0.05 was considered statistically significant.

Relative plasma miRNA expression values (ΔCt) of miR-200a, miR-200b, and miR-141 (Study II) were compared among five study groups (evening and morning samples from patients with and without endometriosis and samples from healthy individuals) with one-way ANOVA following *post hoc* Tukey–Kramer method using SAS 9.4 statistical analysis program. miRNA levels in the evening and morning samples of additionally recruited healthy individuals were compared with paired t-test. P-value < 0.05 were considered as statistically significant for all analyses. Receiver operating characteristic (ROC) curve analysis following the logistic regression modelling was used to evaluate the suitability of circulating miR-200a, miR-200b and miR-141 to discriminate between the evening samples of patients with and without endometriosis and to establish the sensitivity and specificity of each individual miRNA and their combined signature using MedCalc 13.0.4.0.

4.5. mRNA and miRNA profiling from stromal cells (Studies III and IV)

Stromal cells were isolated from endometrial or endometrioma biopsies using fluorescence-activated cell sorting (FACS) as described previously (Krjutskov et al. 2016) with minor modifications for ectopic endometria. Briefly, the biopsies were dissociated in cell culture medium containing 0.5% collagenase IA (Sigma-Aldrich, USA) on a shaking incubator at 37°C, for ~20 min (eutopic endometrium) or ~60 min (ectopic endometrium). The suspension was filtered twice through 50 and 35 µm strainers to separate cells from undigested endometrial tissue fragments. Isolated cells from eu- and ectopic endometria were stained with phycoerythrin-conjugated mouse anti-human CD10 antibody (1:20

dilution, clone HI10a, BD Pharmingen, USA) and were subjected to cell sorting. Total RNA from CD10+ cells was isolated using RNeasy Micro kit (Qiagen, Study III) or miRNeasy Micro kit (Qiagen, Study IV). The quality of RNA was analysed on Bioanalyzer 2100 using RNA 6000 Nano chips (Agilent Technologies, USA; Study III) or 2200 TapeStation RNA ScreenTape (Agilent Technologies; Study IV).

For Study III, library construction and sequencing were performed at the Estonian Genome Center Core Facility (Estonia). mRNA libraries were prepared using TruSeq Stranded mRNA Library Prep Kit (Illumina, USA) per manufacturer's instructions and were sequenced as 100 bp paired end on Illumina HiSeq2500. For miRNA profiling (Study IV), library construction and sequencing were performed at Admera Health LLC (USA). Small RNA libraries were prepared using NEBNext Small RNA Library Prep kit (New England Biolabs, USA) and sequencing was performed with 1×76 bp NextSeq High Output kit on NextSeq 500 platform (Illumina).

4.6. Sequencing data analysis (Studies III and IV)

The quality of sequencing reads was controlled using FastQC program version 0.9.5 (http://www.bioinformatics.bbsrc.ac.uk/projects/fastqc/), read trimming and filtering was performed with cutadapt version 1.8.1 (Martin 2011). Reads that passed the quality control were aligned to the human reference genome using STAR (version 2.4.0j) alignment tool (Dobin *et al.* 2013). Quantification of read counts was performed using HTSeq version 0.6.1 for mRNAs (Study III) and featureCounts v1.5.2 (bioinf.wehi.edu.au/featureCounts) for miRNAs (Study IV).

Differentially expressed genes (DEGs) (Study III) and miRNAs (Study IV) between stromal cells from eutopic and ectopic endometria were identified using edgeR (Robinson *et al.* 2010), DESeq2 (Love *et al.* 2014) and baySeq v2 (Hardcastle 2016) packages. mRNAs or miRNAs were considered as differentially expressed if recognized by at least two out of three methods (adjusted p-values ≤ 0.05).

Biological mechanisms underlying the differentially expressed genes in Study III were analysed using g:Profiler (Reimand *et al.* 2016). Integrated analysis of differentially expressed miRNAs (Study IV) combined with mRNAs (Study III) was used to predict miRNA targets with MAGIA^2 program (Bisognin *et al.* 2012). An additional target prediction analysis for miR-139-5p and miR-375 was conducted using DIANA microT, TargetScan, miRanda and miRDB programs, where genes predicted by at least two programs and that showed differentially expressed levels in Study III were considered as potential targets.

4.7. miRNA transfection (Study IV)

Cultured endometrial stromal cell line ST-T1b (Samalecos *et al.* 2009) was used for miRNA transfection to validate the target genes predicted by bioinformatical analysis for miR-139-5p and miR-375. Cells were cultivated on the cell culture plates prior transfection to reach 70–80% confluency. Cultured cells were transfected with 10 nM miRNA precursors or pre-miR precursor negative control #2 (Thermo Fisher Scientific) via lipotransfection. The experiments were conducted as three separate transfections resulting in a total of eight replicate samples for both miRNAs and eight control samples. The medium was replaced with the respective normal culture medium 24 h later. Expression analyses of predicted miRNA targets for miR-139-5p and miR-375 were performed 48 to 72 h after transfection. RNA was isolated from the cells using innuPREP RNA Mini Kit (Analytik Jena AG, Germany).

4.8. Data validation by qRT-PCR (Studies III and IV)

According to sequencing analysis of Study III, DEGs between eutopic and ectopic stromal cells from complement cascade (*C3, CD55, CFH, A2M* and *SERPINA5*) and cell adhesion-related pathways (*SELP, ESAM, CLDN1* and *CLDN11*) were selected for additional validation by qRT-PCR. Uncultured stromal cells isolated by FACS from nine eutopic and 10 ectopic endometrial tissues were used.

The expression levels of the most up- (miR-139-5p) and downregulated (miR-375) miRNA (Study IV) in ectopic stromal cells compared to eutopic cells were validated by qRT-PCR using paired samples from six individuals. miR-139-5p and miR-375 levels were also quantified from cultured eutopic and ectopic cells. In this experiment, primary cultures of paired endometrial (n=6) and endometrioma (n=6) stromal cells were prepared from frozen tissue biopsies. Single-cell suspensions for primary culture were isolated as described previously (Kasvandik *et al.* 2016). Total RNA from stromal cells were isolated with miRNeasy Micro kit (Qiagen).

cDNA synthesis was conducted with RevertAid First Strand cDNA Synthesis Kit (Thermo Fisher Scientific, USA) for Study III, TaqMan MicroRNA Reverse Transcription Kit (Thermo Fisher Scientific) for miRNA analysis in Study IV and High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems) for mRNA analysis in Study IV. qRT-PCR was performed with 1× HOT FIREPol EvaGreen qPCR Mix Plus (ROX) (Solis BioDyne, Estonia), TaqMan Universal PCR Master Mix, No AmpErase UNG (Thermo Fisher Scientific) or 2 × SYBR Select Master Mix (Applied Biosystems). Relative miRNA/mRNA levels were calculated as described in section 4.4. *SDHA* (Study III), *ACTB* (Study IV, mRNA) or RNU44 and RNU48 (Study IV, miRNA) were used as references for normalisation.

Relative expression levels between eu- and ectopic endometrial stromal cells were compared between the studied groups with two-tailed Student's t-test (mRNA, Study III), Wilcoxon test (miRNA, Study IV) or Mann–Whitney U test (mRNA expression between cells transfected with miRNAs or negative control, Study IV) and p-value < 0.05 was considered significant.

5. RESULTS

5.1. Factors affecting ci-miRNA levels in healthy women and in patients with endometriosis (Studies I and II)

5.1.1. Impact of the menstrual cycle and inter-individual variation on ci-miRNA levels (Study I)

In order to figure out whether ci-miRNA levels are affected by the menstrual cycle in healthy reproductive age women, 375 miRNAs were profiled from plasma samples collected at four different time points during a menstrual cycle. On average, 197 out of 375 tested miRNAs (ranging from 94 to 315 miRNAs per sample with a cut-off value Ct ≤ 36) were detected. miR-451a showed the highest levels in plasma samples. The analysis of circulating plasma miRNAs showed no clustering according to time points within a menstrual cycle (Figure 5).

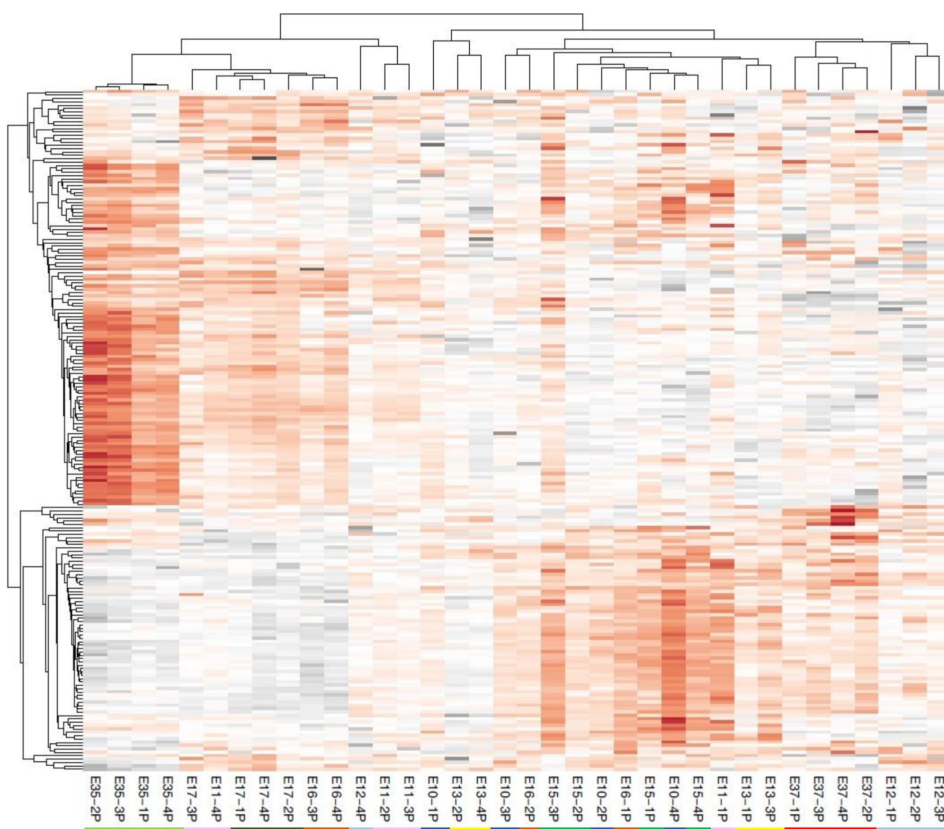

Figure 5. Heatmap of plasma miRNAs in nine healthy women. Numbers E10 – E37 represent the study subjects (coloured lines under the sample code mark different women), 1P- cycle day 1, 2P- cycle day 7, 3P- LH+0, and 4P- LH+7. Red represents miRNAs with higher levels and grey with lower levels compared to the average miRNA level. Figure generated by ClustVis program (https://biit.cs.ut.ee/clustvis/).

No differences in miRNA expression levels or in the number of miRNAs detected within each studied time point were determined [all p-values > 0.0003 (set threshold values for multiple testing)]. Samples showed clustering by study subjects rather than according to menstrual cycle phase (Figure 5). The number of detected miRNAs showed statistically significant differences between the studied individuals (p-value < 0.05).

5.1.2. Comparison of blood and plasma miRNAs (Study I)

miRNAs were also profiled from pooled whole blood samples collected at four different time points from healthy participants. On average, 290 out of 375 miRNAs (ranging from 285 to 296 miRNAs, with a cut-off value Ct ≤ 36) were detected from blood samples. Similar to plasma samples, the most abundantly expressed miRNA in blood was miR-451a. For the comparison of plasma and blood miRNA level differences, all the data from the same sample type were used as technical replicates independent from the menstrual cycle day or the study subject. On average, 296 and 199 miRNAs were detected from blood and plasma samples in this analysis (cut-off value Ct ≤ 36, present in at least 50% of blood or plasma samples), respectively. Out of the determined miRNAs with a used detection criterion, 190 were present in both fractions, 106 were uniquely detected in blood and nine in plasma samples (Figure 6). In addition, 41 miRNAs showed significantly higher and 40 miRNAs lower relative levels in plasma compared to blood samples [FC > 2; all p-values < 0.0003 (set threshold values for multiple testing)].

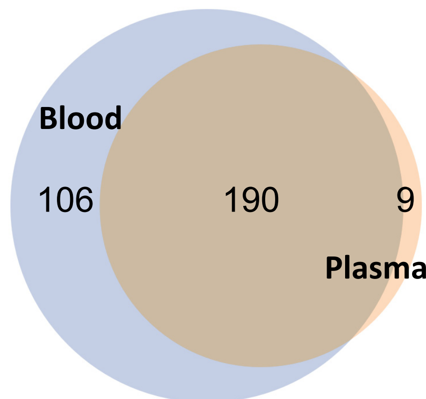

Figure 6. The number of common and unique miRNAs detected from blood (pooled samples from six women at four time points within a menstrual cycle n=4×6) and plasma samples (n=4×9).

5.1.3. Impact of blood collection time on ci-miRNA levels (Study II)

Based on a prior knowledge that some miRNAs have diurnal variation patterns (Shende *et al.* 2011, Figueredo Dde *et al.* 2013), plasma miR-200 family miRNAs were tested in this regard. To determine whether the levels of circulating miR-200a, miR-200b and miR-141 are affected by the sample collection time, the study participants were divided according to the blood sampling time (samples collected in the morning and in the evening). Irrespective of a study group (patients with or without endometriosis), the levels of miR-200 family miRNA showed significant variation between the samples collected in the evening and in the morning, being 1.9-, 1.6-, and 1.6-fold lower in the morning for miR-200a, miR-200b, and miR-141, respectively (all p-values < 0.05). Additional validation with paired samples from healthy volunteers confirmed the circadian fluctuation of miR-200a, miR-200b and miR-141 being over two times lower in the morning (Figure 7; all p-values < 0.05).

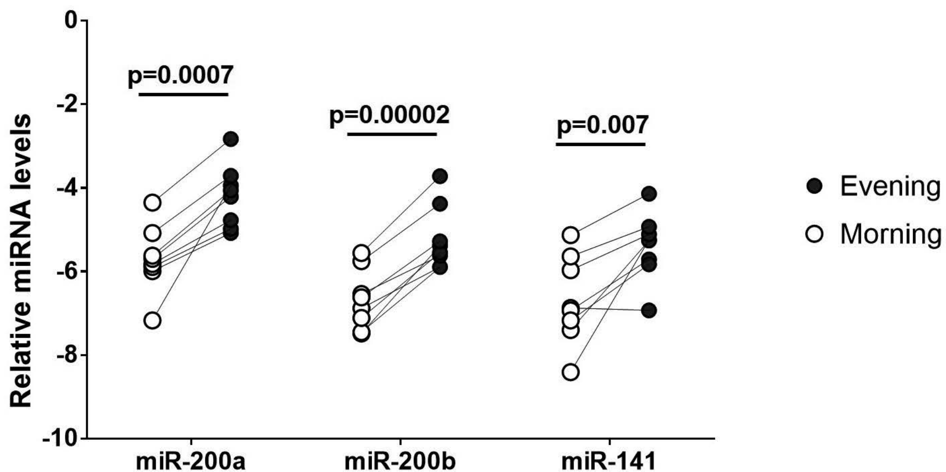

Figure 7. Relative miRNA levels (ΔCt, log2 scale) in paired plasma samples of healthy individuals taken in the evening (n=8) and in the morning (n=8). The levels of miR-200a, miR-200b, and miR-141 were 2.8-, 2.7-, and 2.5-fold lower in the morning compared to evening samples, respectively (all p-values < 0.05). For illustrative purposes, the group means of ΔCt values were multiplied by –1.

5.2. Ci-miRNA profile in endometriosis (Study II)

The miR-200 family miRNAs are differentially expressed in endometriotic lesions compared with eutopic endometria (Ohlsson Teague *et al.* 2009, Filigheddu *et al.* 2010, Hawkins *et al.* 2011). In order to determine whether the miR-200 family miRNAs in circulation also have altered levels between endometriosis patients and controls, the levels of miR-200a, miR-200b and miR-141

were detected from women with endometriosis, surgically confirmed endometriosis-free patients and healthy women. In addition, as data analysis showed circadian fluctuations of studied miR-200-family miRNAs (see section 5.1.3), the blood collection time was taken into account, and circulating plasma miRNA levels were compared among five study groups: morning and evening samples of the patients with endometriosis, morning and evening samples of the patients without endometriosis, and samples of healthy women.

miR-200a and miR-141 levels in the evening samples were significantly lower in women with endometriosis compared to endometriosis-free patients but the levels of miR-200b did not differ among these groups. The levels of miR-200a and miR-200b (but not miR-141) were significantly different between evening samples of the patients with endometriosis and healthy women. The levels of all three studied miRNAs were statistically different between morning samples of endometriosis patients and healthy individuals (all p-values < 0.001), and statistically significant differences were detected in miR-200a and miR-200b levels between morning samples from patients without endometriosis and healthy participants (both p-values < 0.001). The variation between the morning samples of patients and healthy individuals was most likely due to the influence of the blood sampling time as most samples from healthy participants were collected in the evening. miR-200a, miR-200b, and miR-141 levels were significantly lower in patients with stage I–II endometriosis than in endometriosis-free control subjects. Other comparisons are depicted in Table 4.

To evaluate the diagnostic potential of studied plasma miR-200 family miRNAs as endometriosis biomarkers, a ROC curve analysis was performed. Circulating miR-200a showed the best discriminative power to differentiate women with endometriosis from patients with similar complaints but without the disease with a sensitivity of 90.6% and a specificity of 62.5% [Area under the ROC curve (AUC) 0.75]. The combined signature of three miRNAs did not significantly improve the discriminative power for endometriosis patients (sensitivity of 84.4% and a specificity of 66.7%, AUC 0.76).

Table 4. Statistical analysis of miR-200a, miR-200b and miR-141 levels in patients with and without endometriosis and healthy participants.

Study group comparisons	miR-200a	miR-200b	miR-141
Evening samples:			
– Patients with endometriosis *vs* patients without endometriosis	**p=0.002** FC=–1.7	p>0.05	**p=0.024** FC=–1.7
– Patients with endometriosis *vs* healthy participants	**p=0.003** FC =–1.6	**p<0.001** FC=–2.0	p>0.05
– Healthy participants *vs* patients without endometriosis	p>0.05	p>0.05	p>0.05
Evening samples:			
– Stage I–II *vs* patients without endometriosis	**p<0.001** FC=–1.9	**p=0.021** FC =–1.6	**p=0.002** FC =–2.1
– Stage III–IV *vs* patients without endometriosis	**p=0.01** FC=–1.7	p>0.05	p>0.05
Morning samples:			
– Patients with endometriosis *vs* patients without endometriosis	p>0.05	p>0.05	p>0.05
– Patients with endometriosis *vs* healthy participants	**p<0.001** FC =–2.4	**p<0.001** FC =–2.5	**p<0.001** FC =–2.0
– Patients without endometriosis *vs* healthy participants	**p<0.001** FC =–2.3	**p<0.001** FC =–2.7	p>0.05

FC – fold change; values in **bold** are statistically significant between studied groups.

5.3. Eutopic and ectopic stromal mRNA/miRNA profiles (Studies III and IV)

5.3.1. Stroma mRNA profile (Study III)

mRNA sequencing of eutopic and ectopic stromal cells isolated from paired tissue samples from endometriosis patients revealed 1,395 DEGs (FC ≥ 2, adjusted p-values < 0.05) from which 339 were downregulated and 1,056 were upregulated in ectopic cells. Among others, several HOXA genes (*HOXA3, 5, 9, 10, 11, 13*) were upregulated and *EDN1* gene was downregulated in ectopic stroma. “Cell adhesion molecules” and “Complement and coagulation cascades” were among the most enriched KEGG pathways according to the functional annotation analysis of DEGs. The differential expression of selected genes from “Cell adhesion molecules”, including *SELP, ESAM, CLDN1* and

CLDN11 and “Complement and coagulation cascades” pathways, including *C3, CD55, CFH, A2M* and *SERPINA5* was further confirmed by qRT-PCR analyses, showing upregulation in ectopic endometrial stroma (all p-values < 0.05).

5.3.2. Stroma miRNA profile (Study IV)

The same experimental scheme as in Study III was applied to determine the ectopic endometrial stroma miRNA profile. High-throughput sequencing from uncultured paired eutopic and ectopic endometrial stromal cells determined 719 miRNAs in eutopic and 637 miRNAs in ectopic stroma (present in at least 50% of samples). The most abundantly expressed miRNA in both eutopic and ectopic stroma was let-7a-5p. The analysis revealed 149 differentially expressed miRNAs, where 71 miRNAs were more abundantly expressed in eutopic and 78 miRNAs in ectopic stromal cells. miR-139-5p was statistically most significantly upregulated and miR-375 most significantly downregulated miRNA in ectopic stroma and the levels of these miRNAs were confirmed with additional sample set by qRT-PCR (FC = 19 and FC = –42 for miR-139-5p and miR-375, respectively; Figure 8A). miR-139-5p and miR-375 levels were also determined from paired cultured eutopic and ectopic stromal cells, where a slight upregulation of miR-139-5p (FC = 3.2, p-value = 0.03, Figure 8B) in cultured ectopic cells was determined, but no differences were detected in miR-375 levels (p-value > 0.05, Figure 8B). In addition, sequencing analysis revealed the downregulation of miR-200 family miRNAs (miR-200a, miR-200b, miR-200c, miR-141) and upregulation of miR-449a in ectopic cells.

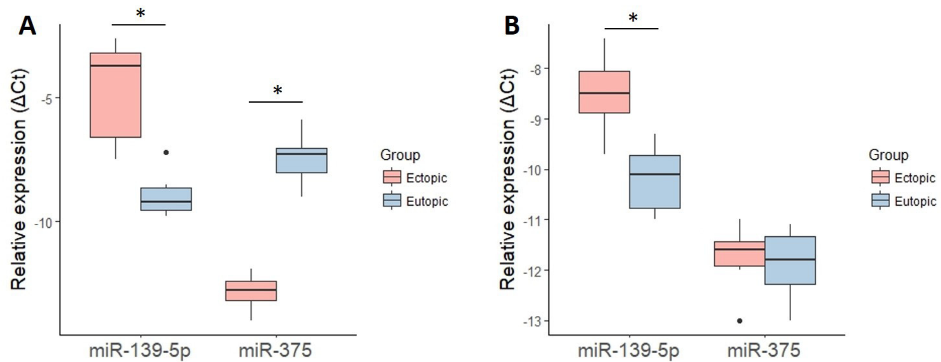

Figure 8. Relative miRNA expression levels (ΔCt, log2 scale) in (**A**) paired uncultured eutopic (n=6) and ectopic (n=6) stromal cells and (**B**) paired cultured eutopic (n=6) and ectopic (n=6) stromal cells. The ΔCt values were calculated as follows: miRNA Ct value − average Ct value of reference genes (RNU44 and RNU48). *p-value < 0.05. Outliers (defined as datapoints outside 1.5 times the interquartile range above the upper quartile and below the lower quartile) are pointed out with black dots. For illustrative purposes, relative expression levels (ΔCt) were multiplied by −1.

5.3.3. Integrated miRNA–mRNA analysis for target identification

To uncover the biological function of altered miRNAs in ectopic stromal cells, a target gene prediction for all differentially expressed miRNAs in Study IV combined with DEGs from Study III was performed using MAGIA[2]. This analysis identified 5,914 significant links (adjusted p-value [q-value] < 0.05) between TFs and their targets (miRNAs or mRNAs) and 1,183 significant links between miRNAs and their targets (miRNA/mRNA or miRNA/TF). Among others, TF *ESR1* was predicted to regulate miR-139-5p from ectopic stromal cells.

With additional analysis, 16 and 19 genes were found as potential targets for miR-139-5p and for miR-375, respectively (Table 5).

Table 5. Common predicted targets for miR-139-5p and miR-375 by DIANA microT, TargetScan, miRANDA and miRDB programs.

Predicted targets for miR-139-5p			
Gene ID	**Gene name**	**Average read count (CPM) in eutopic stroma**	**Average read count (CPM) in ectopic stroma**
ESRRG	Estrogen Related Receptor Gamma	83	6
FBN2	Fibrillin 2	1618	328
HOXA10	Homeobox A10	6376	370
HOXA9	Homeobox A9	957	65
CDH20	Cadherin 20	67	3
GNAO1	G Protein Subunit Alpha O1	349	43
LRFN5	Leucine Rich Repeat And Fibronectin Type III Domain Containing 5	880	49
CKB	Creatine Kinase B	7390	692
GABRA3	Gamma-Aminobutyric Acid Type A Receptor Alpha3 Subunit	308	10
MEX3A	Mex-3 RNA Binding Family Member A	2031	430
PCSK5	Proprotein Convertase Subtilisin/Kexin Type 5	7681	1325
PTPRD	Protein Tyrosine Phosphatase, Receptor Type D	1476	301
RORB	RAR Related Orphan Receptor B	4278	371
TMEM132D	Transmembrane Protein 132D	107	0
PPM1L	Protein Phosphatase, Mg2+/Mn2+ Dependent 1L	1328	235
REM1	RRAD And GEM Like GTPase 1	841	167

Predicted targets for miR-375			
Gene ID	**Gene name**	**Average read count (CPM) in eutopic stroma**	**Average read count (CPM) in ectopic stroma**
CTGF	Connective Tissue Growth Factor	3794	42895
CD200	CD200 Molecule	167	1179
FZD4	Frizzled Class Receptor 4	853	4328
AHR	Aryl Hydrocarbon Receptor	384	2831
GATA6	GATA Binding Protein 6	27	1438
ZFPM2	Zinc Finger Protein, FOG Family Member 2	165	1022
EDN1	Endothelin 1	65	932
NCAM1	Neural Cell Adhesion Molecule 1	47	957
IL1RAP	Interleukin 1 Receptor Accessory Protein	116	449
DUSP6	Dual Specificity Phosphatase 6	886	5846
EBF3	Early B-Cell Factor 3	2	144
RBM47	RNA Binding Motif Protein 47	12	87
ARAP2	ArfGAP With RhoGAP Domain, Ankyrin Repeat And PH Domain 2	27	231
SHANK3	SH3 And Multiple Ankyrin Repeat Domains 3	685	5515
ARL4C	ADP Ribosylation Factor Like GTPase 4C	404	2483
EXOC6	Exocyst Complex Component 6	119	1019
IFIT2	Interferon Induced Protein With Tetratricopeptide Repeats 2	127	526
PTPRM	Protein Tyrosine Phosphatase, Receptor Type M	553	5389
WBP1L	WW Domain Binding Protein 1 Like	1353	4885

Genes in **bold** were selected for validation by qRT-PCR from cells transfected with miR-139-5p or miR-375. CPM – count per million.

To examine the impact of miR-139-5p and miR-375 on target gene expression in stromal cells, the endometrial stromal cell line ST-T1b was transfected with selected miRNA precursors or negative control precursor. The expression of seven (*CDH20, ESRRG, FBN2, HOXA9, HOXA10, LRFN5, GNAO1*) and 10 (*EDN1, ZFPM2, GATA6, FZD4, AHR, CD200, CTGF, DUSP6, IL1RAP, NCAM1*) potential target genes for miR-139-5p and miR-375 were selected for validation by qRT-PCR (Table 5). The overexpression of miR-139-5p by transfection resulted in 2.1-fold and 1.8-fold downregulation of *HOXA9* and *HOXA10* expression compared to control samples, respectively (Figure 9A and

B; all p-values < 0.05). The overexpression of miR-375 resulted in a 1.9-fold downregulation of *EDN1* gene expression (Figure 9C; p-value < 0.05). The expression of the other tested target genes did not differ between the miRNA or negative control transfected cells (all p-values > 0.05).

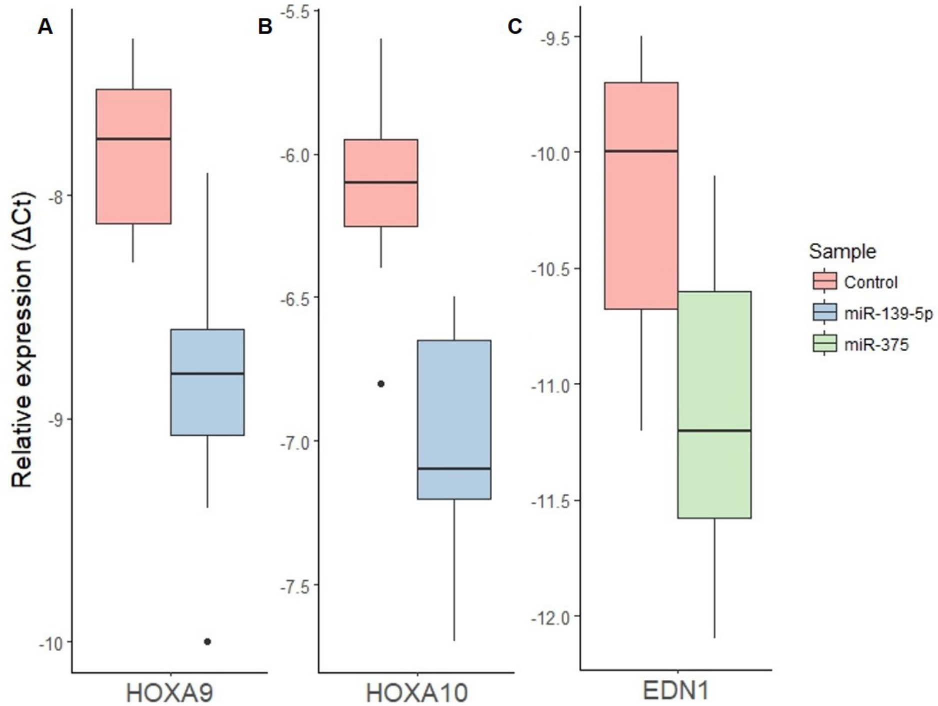

Figure 9. Relative gene expression levels (ΔCt, log2 scale) in ST-T1b stromal cell line transfected with miR-139-5p precursor (blue), miR-375 precursor (green) or negative control (red). The experiments were conducted as three separate transfections, in a total of eight replicate samples for both miRNAs and eight control samples. All depicted values were statistically significant (p-values < 0.05) between miRNA precursors and control transfected cells resulting in 2.1-fold and 1.8-fold downregulation of *HOXA9* and *HOXA10* expression in cells transfected with miR-139-5p and 1.9-fold downregulation of *EDN1* expression in cells transfected with miR-375. Error bars in boxplot denominate 1.5 × interquartile range, outliers are pointed out with black dots. For illustrative purposes, the group means of ΔCt values were multiplied by –1.

6. DISCUSSION

Aberrant miRNA expression may contribute to the endometriosis pathogenesis by modifying the gene expression in endometriotic cells. Also, ci-miRNAs hold a potential as non-invasive biomarkers for disease diagnostics. We showed that ci-miRNA levels are affected by several biological factors and the detection of gene and miRNA expression levels in endometriotic tissues may be influenced by the cellular composition of the investigated specimens. In the following sections, these findings are discussed in the context of the current state of miRNAs as biomarkers and in the context of endometriosis pathogenesis.

6.1. Factors influencing ci-miRNA levels

Hormonal fluctuations across the menstrual cycle affect the whole female body. It is known that miRNAs regulate some of the cellular processes during the cyclic changes in the endometrium and other tissues (Kuokkanen *et al.* 2010, McBride *et al.* 2012). Therefore, it was reasonable to assume that menstrual cycle confounds ci-miRNA profiling in women of reproductive age. Indeed, cyclic changes in ci-miRNA levels have been observed in a few studies. Cho *et al*. found significantly higher levels of let-7 family miRNAs in the secretory phase compared to the proliferative phase in serum of women with endometriosis, while no differences were observed in the control group (Cho et al. 2015). Interestingly, no differences in plasma miRNA levels between the menstrual cycle time points were found from healthy individuals in Study I. The most recent study on ci-miRNAs in endometriosis showed that there are some plasma miRNAs that fluctuate during the menstrual cycle in both patients with endometriosis and healthy women (Nisenblat *et al.* 2019); however, the authors agree with the results of our Study I that the overall plasma miRNA profile in healthy women remains stable throughout the menstrual cycle. This indicates that menstrual cycle is not a major confounding factor to influence miRNA levels in plasma and ci-miRNAs are therefore suitable markers in reproductive age women.

We cannot rule out that the significant changes in plasma miRNA levels throughout the menstrual cycle were not observed as only a particular subset of miRNAs (375 miRNAs) were investigated or because of the relatively small number of samples were analysed in our study. Moreover, we noted a significant inter-individual difference in the numbers of plasma ci-miRNAs and distinct samples from the same woman tended to cluster together. Thereby, it is likely that the individual-specific differences in ci-miRNA signatures masked the possible small fluctuations in miRNA levels throughout the cycle. Ci-miRNA profiles within individuals is influenced by an array of external factors, such as age, gender, ethnicity, and BMI (Duttagupta *et al.* 2011, Huang *et al.* 2011, Sredni *et al.* 2011, Ameling *et al.* 2015). However, it is unlikely that these

factors had significant effect on miRNA levels in our study as recruited healthy volunteers were of the same ethnic origin, similar age and BMI meaning that other aspects must be behind the inter-individual differences. Nevertheless, the variation between individuals is an important element to consider when studying ci-miRNAs.

Blood cells are leading contributors to ci-miRNAs in plasma, and inevitably, ci-miRNA levels are directly influenced by blood cell counts at the time of sampling (Pritchard *et al.* 2012). In this study, we showed that the majority of miRNAs present in whole blood and plasma overlap, but there were also a number of miRNAs unique to a sample type. In addition, the relative levels of several miRNAs were higher in plasma compared to blood samples, suggesting that some ci-miRNAs in plasma originate from cells other than blood. It has been widely speculated that miRNAs from various cells and tissues besides blood enter the circulation (Mitchell *et al.* 2008). Indeed, exosomal miRNAs from placental origin can be detected from maternal blood plasma (Chim et al. 2008, Luo et al. 2009). However, the quantity of ci-miRNAs derived from other tissues is extremely low and cannot be detected from whole blood samples due to high abundance of blood cell miRNAs in samples which overshadows the circulating cell free miRNAs. Therefore, plasma is probably more suitable sample type for studying ci-miRNAs as biomarker candidates as it is more likely to find miRNAs originating from other cells and tissues besides blood cells.

In addition to the inter-individual variation and cellular composition of blood, the timing of sample collection may affect the ci-miRNA levels as was demonstrated in Study II. We observed lower levels of plasma miR-200a, miR-200b and miR-141 in samples collected in the morning compared to those drawn in the evening both in endometriosis patients and in healthy volunteers. Although the healthy individuals were of heterogeneous age and BMI and some of the participants used hormonal medications at the time of participation, the effect of blood sampling time on miR-200 family ci-miRNA levels was observed in all of them, confirming that this was truly a circadian rhythm effect.

The levels of many genes and proteins vary according to a circadian cycle (reviewed in Takahashi 2017). In fact, it is estimated that over 40% of mammalian transcripts fluctuate daily (Zhang *et al.* 2014). However, around half of the rhythmically expressed proteins do not exhibit cyclic patterns on mRNA levels (Reddy *et al.* 2006). Therefore, circadian expression patterns in mammals are mainly regulated via post-transcriptional level (Kojima *et al.* 2011) or through epigenetic changes (Azzi *et al.* 2014). Also, miRNAs have a role in the post-transcriptional level regulation of the circadian clock genes (Cheng *et al.* 2007, Kadener *et al.* 2009, Shende *et al.* 2011).

Circadian variation of ci-miRNAs has been demonstrated in a few studies (Shende *et al.* 2011, Heegaard *et al.* 2016). Heegaard *et al*. investigated male volunteers and demonstrated that a third (26 out of 79) of measurable plasma miRNAs exhibited a rhythmic behaviour (Heegaard *et al.* 2016), and some of these miRNAs target known clock genes. This study did not report the cyclic variation of miR-200 family miRNAs which could be due to technical aspects

as the used methodology was unable to consistently detect miR-200 family miRNAs in plasma. Also, we cannot exclude that miR-200 family miRNAs have distinct expression patterns in blood plasma of males and females.

Awareness of oscillations in potential biomarker levels during the circadian cycle is an important factor to consider if developing diagnostic tests. To the best of our knowledge, none of the studies aiming to discover endometriosis miRNA biomarkers have taken this variation into account.

6.2. Suitability of ci-miRNAs as endometriosis biomarkers

Endometriosis is a disease that still lacks reliable diagnostic options other than laparoscopic surgery and ci-miRNAs have been proposed as a new potential source of non-invasive biomarkers. In Study II, we demonstrated that miR-200a and miR-141 levels in blood plasma are significantly lower in endometriosis patients than in laparoscopically confirmed disease-free women.

The source of cell-free miRNA level variations in circulation is difficult to determine. We have previously demonstrated that miR-200a, miR-200b and miR-141 are not differentially expressed in the endometrium of women with and without endometriosis (Saare *et al.* 2014), so the endometrium is likely not the tissue of origin for the ci-miR-200 alterations in endometriosis. The miR-200 family miRNAs are downregulated in endometriotic lesions compared with eutopic endometria (Ohlsson Teague *et al.* 2009, Filigheddu *et al.* 2010, Hawkins *et al.* 2011) and we confirmed the lower levels of miR-200 family miRNAs in ectopic compared to eutopic stromal cells in Study IV. As miR-200 miRNAs were downregulated in ectopic tissues, the ectopic lesions cannot be the source of ci-miRNA-200 differences in plasma. Instead, these alterations are probably caused by the systemic changes throughout the body by the presence of endometriosis.

Several reports have been published demonstrating ci-miRNAs as potential endometriosis biomarkers (Table 2); however, the overlap between the findings has remained minimal and only a few of the proposed differentially expressed miRNAs have been common among the studies. For example, miR-139-3p has been shown as downregulated in endometriosis patients in three studies (Wang *et al.* 2013, Hsu *et al.* 2014, Nisenblat *et al.* 2019). Decreased levels of miR-200a-3p and miR-141-3p in endometriosis patients described by Study II have not been determined previously. As discussed earlier, several factors other than the disease, such as inter-individual variation (Study I) and circadian cycle (Study II), contribute to ci-miRNA level alterations. The effect of circadian rhythm on miR-200 levels was even more prominent than the disease status; however, none of the ci-miRNA studies in endometriosis have taken these factors into account. Therefore, biological factors, as well as technical differences in study design are the likely causes for the discrepancies among discovered findings.

Previously published studies have reported endometriosis ci-miRNA candidate markers with a broad range of diagnostic accuracy (Agrawal *et al.*

2018). Although miR-125b-5p was able to distinguish endometriosis patients from disease-free women with a sensitivity of 100% and a specificity of 96% in one study (Cosar *et al.* 2016), the majority of the ci-miRNAs alone exhibit rather moderate accuracy with a sensitivity around 80% (Agrawal *et al.* 2018). However, using a combination of several miRNAs usually helps to improve the diagnostic potential of ci-miRNAs. For example, miR-122 (sensitivity 80%/ specificity 76%), miR-145 (sensitivity 70%/specificity 96%), miR-199a (sensitivity 78%/specificity 76%) and miR-542-3p (sensitivity 80%/specificity 92%) alone had moderate diagnostic potential, but the combination of these ci-miRNAs reached a sensitivity and a specificity of 93% and 96%, respectively (Wang *et al.* 2013). Out of the three miR-200 family members investigated in Study II, miR-200a indicated the best discriminative power to distinguish women with endometriosis from patients with similar complaints but without the disease (sensitivity 90.6%, specificity 62.5%). The diagnostic accuracy did not improve, when a combination of all three miRNAs was used. miR-200a and miR-200b are transcribed from the same pri-miRNA molecule and their levels are highly correlated (data from miRBase.org), therefore, the addition of miR-200b did not advance the accuracy of the test. Although belonging to the same family of miRNAs, miR-141 is transcribed from a different molecule; however, the diagnostic potential of miR-141 alone as endometriosis biomarker was low and the addition of miR-141 to the analysis model decreased the disease-prediction power. Based on the results of Study II, ci-miR-200 family miRNAs are not sufficient to set a clinical diagnosis; however, in combination with other markers or imaging techniques these miRNAs could be used to yield improved precision in diagnosing endometriosis.

Furthermore, most proposed ci-miRNA biomarkers are not specific to a disease. It has even been suggested that a truly disease-specific ci-miRNA does not exist as many of the proposed miRNA biomarkers for one disease have been found to be associated with a variety of other conditions (Haider et al. 2014). Moreover, miRNA levels usually do not differ drastically between cases and controls and the confounding factors challenge the use of ci-miRNAs as diagnostic biomarkers. The confounding factors revealed in our studies pointed out the importance of considering inter-individual differences and circadian variation in ci-miRNA studies in humans. Taken together, the author of the current thesis agrees with the recent suggestion that ci-miRNAs as diagnostic biomarkers for endometriosis have a limited potential (Nisenblat *et al.* 2019).

6.3. The role of miRNAs in endometriotic tissues

In recent years, the impact of cellular heterogeneity of tissue samples on endometriosis studies has started to receive attention (Saare *et al.* 2017). Tissues are comprised of several cell types and each cell population exhibits unique pattern of miRNA expression. Despite of this, the majority of studies on endometriosis have used entire biopsied lesion samples where the proportion of endometrial

epithelial and stromal cells is variable, and the surrounding tissue may remarkably contribute to detectable miRNA patterns (Saare *et al.* 2014). Therefore, tissue heterogeneity can partially explain the discordant results obtained in previous endometriosis miRNA studies where whole lesion biopsies from distinct anatomical locations were compared to eutopic endometrium. If studies with similar design are compared, the overlap between the results increases significantly. For example, the findings of studies conducted with endometrioma samples only (Filigheddu *et al.* 2010, Hawkins *et al.* 2011, Braza-Boils *et al.* 2014, Zhao *et al.* 2018) have higher overlap with each other (Saare *et al.* 2017) than all the miRNA studies on endometriosis combined.

In order to overcome the problem of tissue heterogeneity, we used flow cytometry to retrieve a purified fraction of uncultured CD10+ stromal cells for the detection of endometriosis-specific mRNA and miRNA changes in eutopic and ectopic endometrial cells (Studies III and IV, respectively). All studied ectopic stromal cells were isolated from endometrioma samples to exclude the variance of expression patterns due to anatomical location of lesions. Our studies on purified stromal cells showed over 1,300 DEGs and 149 differentially expressed miRNAs in ectopic stromal cells compared to eutopic stroma. The analysis of DEGs confirmed the previously published associations between endometriosis and complement cascade genes but also added novel candidate genes involved in the pathogenesis of the disease. The gene expression analysis also paved the way for predicting target molecules for differentially expressed miRNAs in ectopic tissues. The integrated analysis of differentially expressed mRNAs and miRNAs in ectopic tissues revealed sophisticated regulatory networks between TFs, genes and miRNAs. Perhaps the most interesting finding from the combined analysis of differentially expressed transcripts was the regulatory effect of *ESR1* on miR-139-5p that was the most highly upregulated miRNA in ectopic stromal cells. By target gene analysis and transfection experiments, we showed that miR-139-5p inhibits *HOXA10* and *HOXA9* gene levels in stromal cells. Although miR-139-5p has not been associated with endometriosis before, the regulatory link between miR-139-5p and *HOXA10* has been previously demonstrated (Liu *et al.* 2018). In endometrial cancer cells, miR-139-5p targets the *HOXA10* transcript and suppresses endometrial cancer cell growth and migration (Liu *et al.* 2018). Moreover, *ESR1* is known to regulate *HOXA10* levels (Taylor *et al.* 1998) suggesting a refined regulatory feedback loops between *ESR1*, *HOXA10* and miR-139-5p.

Furthermore, *HOXA10* is downregulated in ovarian endometriomas and peritoneal endometriotic lesions compared to eutopic endometrium (Browne and Taylor 2006, Zheng *et al.* 2018). *HOXA10* and *HOXA9* are highly expressed in the endometrium and the expression is influenced by the menstrual cycle where *HOXA10* levels peak during the window of implantation (Taylor *et al.* 1998). In women with endometriosis, endometrial HOXA10 expression is lower during this time-frame (Taylor *et al.* 1999), but the levels are restored after the removal of endometriotic lesions (Celik *et al.* 2015), indicating the role of this gene in endometriosis-associated infertility.

It has to be mentioned that many of the differentially expressed transcripts in our study have already been reported in endometriosis studies investigating whole eutopic and ectopic tissues, suggesting that if a certain miRNA or gene is highly present or completely absent in ectopic endometrial cells, the surrounding tissue does not hinder the detection of their differential expression. There are certain miRNAs that have been constantly reported as dysregulated between eutopic and ectopic tissues including miR-200-family (miR-200a, miR-200b, miR-200c and miR-141), miR-196b-5p, miR-183-3p, miR-34c-5p, miR-375 miR-202-3p and miR-202-5p (Saare *et al.* 2017) which were also seen in Study IV and therefore, the abovementioned miRNAs most likely do contribute to the endometriosis pathogenesis.

One of the common dysregulated miRNA, miR-375, was the most prominently downregulated in endometriotic stromal cells in Study IV. With bioinformatical and experimental approaches, we found that *EDN1* gene expression is probably regulated by miR-375. Endothelin-1 (ET-1, encoded by the *EDN1* gene) is primarily known as a vasoconstrictor secreted by endothelial cells. The association of *EDN1* gene and endometriosis has been proposed previously as the cystic fluid of endometriomas contains a higher amount of ET-1 compared to ovarian cysts other than endometriomas (Yoshino *et al.* 2018). In mice, the inhibition of ET-1 was effective in decreasing endometriosis-related pain (Yoshino *et al.* 2018). ET-1 also supports the survival and migration of mesenchymal stem cells (Pourjafar *et al.* 2016), that are proposed to be involved in endometriosis development (Barragan *et al.* 2016, Cousins *et al.* 2018). Thus, the regulatory link between miR-139-5p and *EDN1* gene could be used as potential therapy option for endometriosis-related pain or to prevent the migration of endometrial mesenchymal stem cells to the ectopic locations.

Another interesting phenomenon was that we observed the downregulation of miR-375 in ectopic stroma only in FACS-isolated uncultured cells and not in *in vitro* culture. This finding emphasises the importance of performing the expression studies in uncultured cells as culturing may considerably alter the miRNA expression levels.

An additional issue needs to be acknowledged in association with differential gene and miRNA expression in eutopic and ectopic endometrial tissues. As endometrium undergoes cyclic growth and degeneration, the menstrual cycle affects gene and miRNA expression in endometrial tissues in healthy women and also in patients with endometriosis (Kuokkanen *et al.* 2010, Altmae *et al.* 2017, Xu *et al.* 2017, Rekker *et al.* 2018). The samples used in Studies III and IV were all from proliferative menstrual cycle phase, but we noticed that several genes and miRNAs, shown to be regulated by monthly cyclic variation (e.g. miR-200 and miR-449 families; *GPX3, DKK1, SERPING1, MMP* genes) exhibited differential expression between eutopic and ectopic stroma in our studies. These may be true differences between cells from eutopic and ectopic locations but could also be related to the fact that in ectopic tissues the menstrual cycle is not synchronised with eutopic endometrium (Sohler *et al.* 2013). If endometrial cells from ectopic locations do not respond to hormonal changes in the same

way as endometrial cells in the uterus, hormonal dysregulation in lesions may be the source of altered gene and miRNA levels in endometriosis. Therefore, at least some expression differences reported in Studies III and IV may be caused by the menstrual cycle anomalies in ectopic tissues and are not directly related to pathogenetic processes in lesions.

To summarise, our studies demonstrated that investigating purified stromal cells allows to reveal many DEGs, miRNAs and their interactions that are most likely involved in endometriosis pathogenesis. Furthermore, such kind of study design enables to avoid the detection of expression changes possibly caused by the non-endometrial cells in ectopic biopsies which could be the case if studying whole-tissue specimens.

6.4. Future perspectives

Given the heterogenous phenotype of endometriosis, universal biomarker that can accurately diagnose all forms of the disease probably does not exists. Although ci-miRNAs have some capacity to discriminate endometriosis patients from individuals with similar symptoms, their diagnostic potential as stand-alone marker remains low. In my opinion, further studies of ci-miRNAs at the current form and design should be refrained. Recent developments in miRNA detection with amplification-free technologies and without the need for extensive RNA isolation from biofluids (Jin *et al.* 2015, Foye *et al.* 2017, Cao *et al.* 2019) may simplify the applicability of ci-miRNAs as true biomarkers for the disease. Moreover, proper stratification of patients with different subtypes of endometriosis is necessary to allow personalised approach for endometriosis diagnostics considering all aspects of the disease. Yet, in order to quantify ci-miRNAs from different types and severity stage of endometriosis separately, larger sample size is needed and, for this reason, extensive collaboration with highly standardised protocols between research groups is vital. Nonetheless, the inter-individual and biological variation of ci-miRNA patterns remains; thus, the adoption of miRNA testing in clinical settings remains challenging.

Future research focus involving endometriotic tissue samples should be projected to obtaining specific single cells or cell populations within a lesion. Powerful methods that enable the analysis of the miRNome of a single cell or even co-sequencing of miRNAs and mRNAs within the same cell have been developed (Wang et al. 2019) and endometriosis studies would greatly benefit from these innovative techniques. Also, tissue samples of the endometriotic lesions from different locations should be studied separately in prospective studies. The analysis of separate cell populations within tissues will hopefully help to detect the true biological and physiological processes that are behind the disease pathogenesis. That, in turn, will facilitate the discovery of specific biomarkers and therapeutic targets for endometriosis, necessary to shorten the time to diagnosis and to provide more efficient patient care.

7. CONCLUSION

Based on the results of the current thesis, following conclusions can be made:

1. There are no substantial differences in studied ci-miRNA levels throughout the menstrual cycle, but the inter-individual variations in ci-miRNA patterns does exist.
2. Ci-miRNAs, including miR-200a, miR-200b and miR-141, have differential levels between endometriosis patients and controls. Plasma miR-200 family miRNAs are also affected by the circadian cycle being lower in the morning compared to evening samples and the effect of circadian cycle for studied miRNA levels was more prominent than the disease status. Due to the biological variation of ci-miRNAs, the potential of these molecules as diagnostic markers is limited.
3. Based on the integrated analysis of differentially expressed transcripts, we propose two molecular mechanisms involved in endometriosis pathogenesis, where, firstly, the regulatory link between *HOXA10* and miR-139-5p is potentially involved in endometriosis-associated infertility. Secondly, the lower levels of miR-375 in ectopic stromal cells may lead to higher levels of *EDN1* in lesions, that can be associated with endometriosis-related pain or be involved in mesenchymal stem cell actions outside the uterus. However, further functional studies are necessary to prove the connections between the proposed regulatory networks and endometriosis pathogenesis.

8. SUMMARY IN ESTONIAN

mikroRNA-de potentsiaalne roll endometrioosi patogeneesis ja diagnostikas

Endometrioos on sage günekoloogiline haigus, mis esineb ligikaudu 5–10% viljakas eas naistel. Endometrioosi korral paikneb emaka sisepinda vooderdav limaskest – endomeetrium – väljaspool emakaõõnt, moodustades koldeid väikevaagna organitel. Endometrioidne kude allub organismi tsüklilistele muutustele ning kasvab ja irdub perioodiliselt sarnaselt endomeetriumile, mis võib põhjustada tugevaid valusid menstruatsiooni ajal. Endometrioosiga võib kaasneda ka viljatus. Vaatamata ulatuslikele uuringutele antud valdkonnas on endometrioosi diagnoosimise võimalused piiratud ning ainus viis haiguse lõplikuks kinnitamiseks on laparoskoopiline operatsioon. Ebaspetsiifiliste haigussümptomite tõttu suunatakse diagnostilisele operatsioonile sageli ka endometrioosile viitavate kaebustega naised, kellel haigust ei leita. Hetkel loetaksegi üheks prioriteetseimaks endometrioosi uurimissuunaks mitte-invasiivsete biomarkerite avastamist. Haigusseoseliste markerite otsingul kasutatakse sageli erinevaid proteoomika ja transkriptoomika meetodeid ning viimasel ajal on uute endometrioosi kandidaatmarkeritena kerkinud esile mikroRNA-d (miRNA-d).

MiRNA-d on lühikesed umbes 18–22 nukleotiidi pikkused üheahelalised RNA molekulid, mis reguleerivad geeniekspressiooni transkriptsiooni-järgselt. Varieeruvusi normaalsest miRNA ekspressioonimustrist on täheldatud mitmete patoloogiate, sealhulgas günekoloogiliste haiguste, pahaloomuliste kasvajate ja viljatuse korral. Rakuvabad miRNA-d on tuvastatavad erinevatest kehavedelikest, näiteks vereplasma, uriin ja sülg, mistõttu on miRNA-sid võimalik hõlpsalt testida. Samuti on rakuvabad tsirkuleerivad miRNA-d bioloogilistes proovides väga stabiilsed ja vastupidavad erinevatele mõjutustele. Samas näib, et nende taset mõjutavad normaalsed füsioloogilised muutujad, sealhulgas vanus ja sugu ning ööpäevane rütm, mida tuleb arvesse võtta, kui hinnata miRNA-de kui haigusseoseliste markerite potentsiaali diagnostikaks. Reproduktiivses eas naiste puhul on vajalik ka teave miRNA-de normaalsest varieeruvusest menstruaaltsükli lõikes.

Mitte-invasiivse diagnostika puudumine võib osaliselt olla põhjustatud ka ebapiisavatest teadmistest endometrioosi patogeneesi-mehhanismide kohta. Uuringud on näidanud, et miRNA-del on roll haiguse arengus ja püsimajäämises. Seniste uuringute tulemused on paraku vastuolulised, kuna sageli kasutatakse endometrioosi uuringutes laparoskoopilise operatsiooni käigus eemaldatud koldeid ning võrreldakse nende miRNA ekspressiooniprofiili endomeetriumi omaga. Kolde biopsiad on heterogeense rakulise koostisega, moodustudes peamiselt endomeetriumile iseloomulikest epiteeli ja strooma rakkudest, segatuna ümbritsevast koest pärinevate rakkudega. Koe heterogeensus takistab kolde rakkudele iseloomulike miRNA mustrite tuvastamist, mis on ilmselt üheks põhjuseks senistele vastuolulistele tulemustele. Leidmaks muutusi, mis võimal-

davad endomeetriumi rakkude ellujäämist ja kasvamist väljaspool emakat, tuleks välja selgitada iga rakutüübi omane molekulaarne profiil eraldi.

Uurimistöö eesmärgid

Käesoleva töö eesmärgiks oli välja selgitada miRNA-de roll endometrioosi patogeneesis ja hinnata antud molekulide sobivust mitte-invasiivsete biomarkeritena. Uuringu alameesmärgid olid sellest tulenevalt järgmised:

a) hinnata tsirkuleerivate miRNA-de profiili tervetel naistel menstruaaltsükli vältel (uuring I);
b) määrata kindlaks plasma miR-200a, miR-200b ja miR-141 tasemed endometrioosiga patsientidel ning hinnata antud markerite sobivust endometrioosi diagnostikas (uuring II);
c) välja selgitada, kas endometrioosiga patsientide endomeetriumi ja endometrioosikollete stroomarakkude miRNA ekspressioonimuster on erinev ning, kõrvutades tulemusi geeniekspressiooni andmetega, määrata kindlaks miRNA-de regulatsioonimehhanismid endometrioosikolde rakkudes (uuringud III ja IV).

Materjal ja metoodika

Uuringusse värvati Tartu Ülikooli Kliinikumi naistekliinikus ja Elite kliinikus endometrioosi kahtluse või viljatuse tõttu laparoskoopilisele operatsioonile suunatud patsiendid. Endometrioosi diagnoos kinnitati kirurgiliste ja histoloogiliste leidudega. Uuritavad, kellel operatsiooni käigus endometrioosi ei tuvastatud, määratleti kontroll-grupiks. Kõikidelt värvatud patsientidelt võeti operatsiooni eelselt vereproov, lisaks koguti uuringus osalejatelt Pipelle kogumiskateetrit kasutades endomeetriumi biopsia ning endometrioosiga patsientidelt operatsiooni käigus eemaldatud endometrioosi kolded. Kolletest kasutati antud uuringus vaid munasarja endometrioome.

Lisaks kaasati uuringutesse I ja II vabatahtlikud naised, kes määratlesid ennast tervetena. Ühelgi tervetest vabatahtlikest ei olnud varem esinenud endometrioosi ega autoimmuunseid haiguseid. Tervetelt naistelt koguti vereproovid. Kaks naist täiendavast uuringurühmast (uuring II) kasutasid osalemise ajal hormonaalseid rasestumisvastaseid ravimeid. Ükski teine naine ei olnud tarvitanud hormonaalseid preparaate uuringule eelnenud kolme kuu jooksul. Kõik uuringus osalenud naised allkirjastasid uuringus osalemise nõusolekuvormi.

Järgnevalt on toodud kasutatud metoodikad uuringute kaupa:

Uuring I: miRNA profiili varieeruvuse uurimiseks menstruaaltsükli vältel kaasati terved naised (n=9), kellelt koguti vereproovid neljas ajapunktis – menstruaaltsükli esimesel ja seitsmendal päeval, luteiniseeriva hormooni (LH) taseme tõusu toimumise päeval ning seitse päeva peale LH taseme tõusu toimumist. Kogutud vere ja eraldatud plasma proovidest miRNA ekspressioonimustri

määramine toimus Exiqon analüüsiplatvormi abil, mis võimaldab korraga määrata 375 miRNA tasemed kasutades kvantitatiivset reaal-aja PCR-i (qRT-PCR).

Uuring II: Endometrioosi-spetsiifiliste biomarkerite leidmiseks võrreldi endometrioosiga (n=61) ja kontrollgrupi naiste (n=35) ning tervete vabatahtlike (n=30) vereplasmast määratud miR-200 perekonna miRNA-de (miR-200a, miR-200b ja miR-141) profiili qRT-PCR meetodi abil. Lisaks määrati samade miRNA-de tase uuringusse kaasatud kaheksa terve naise vereplasmast, kellelt koguti proovid õhtul (n=8) ning sellele järgneval hommikul (n=8), et kindlaks teha, kas miR-200 perekonna miRNA-de tase kõigub ööpäeva lõikes.

Uuringud III ja IV: Endometrioosiga patsientide endomeetriumi ja endometrioosikollete stroomarakkude miRNA ja geeniekspressioonimustri määramiseks kasutati süvasekveneerimist. Selleks eraldati endometrioosiga naiste endomeetriumist ja endometrioosikolletest voolutsütomeetriaga CD10+ stroomarakud, millest eraldatud RNA sekveneeriti. Sekveneerimistulemused valideeriti qRT-PCR-iga. Endometroosikolde rakkudes erinevalt avaldunud miRNA-de funktsiooni kindlaksmääramiseks teostati *in vitro* transfektsioonikatsed ning miRNA-de sihtmärkgeenide ekspressioonitasemeid transfekteeritud rakkudes hinnati qRT-PCR abil.

Tulemused

Antud doktoritöö raames tehtud tööde tulemused võib kokku võtta järgnevalt:

1. Viljakas eas tervete naiste tsirkuleerivate miRNA-de profiilis ei esinenud menstruaaltsükli erinevates ajapunktides statistiliselt olulisi arvulisi ega ka taseme muutusi. Samas avastasime, et indiviidide vaheline tsirkuleerivate miRNA-de varieeruvus oli statistiliselt oluline (p-väärtus < 0.05). Määrasime miRNA-de profiili ka täisverest ning võrdlesime seda plasma miRNA-de tasemetega. Analüüsi tulemusena leidsime 190 miRNA-d, mis olid detekteeritavad mõlemast fraktsioonist, 106 olid unikaalsed vereproovile ja üheksa plasmale. Lisaks olid 41 miRNA-d oluliselt kõrgema ja 40 miRNA-d madalama tasemega plasmas võrreldes vereproovidega.
2. Endometrioosi mitte-invasiivsete biomarkerite uuringu tulemustest selgus, et tsirkuleerivate miR-200a ja miR-141 tasemed olid õhtul kogutud vereproovides endometrioosiga naistel oluliselt madalamad kui kontroll-grupi patsientidel, kuid miR-200b tase ei erinenud nende rühmade vahel. Parima diagnostilise väärtusega oli miR-200a, mille tundlikkus ja spetsiifilisus endometrioosi tuvastamisel olid vastavalt 90,6% ja 62,5%. Huvitava leiuna tuli välja, et kõigi kolme uuritud vereplasma miRNA tasemed olid nii patsientidel kui ka tervetel indiviididel õhtul kogutud vereproovides umbes kaks korda kõrgema tasemega kui hommikul kogutud proovides (kõik p-väärtused < 0.05) ning ööpäevase rütmi mõju uuritud tsirkuleerivate miRNA-de tasemetele oli suurem kui haigusseoseline efekt.

3. Endometrioosiga patsientide endomeetriumi ja endometrioosikollete strooma-rakkude transkriptoomi võrdluse tulemusena leiti 1395 erinevalt ekspresseeritud geeni ning 149 erineva tasemega miRNA-d. Uuringutes III ja IV saadud andmete integreeritud analüüs näitas olulisi ja kompleksseid seoseid aberrantselt ekspresseeritud transkriptsioonifaktorite, geenide ja miRNA-de vahel. Kõige erinevamalt olid kolde ja endomeetriumi vahel ekspresseeritud raku adhesiooni ning komplemendi rajaga seotud geenid. miR-139-5p oli statistiliselt kõige rohkem üles-reguleeritud ja miR-375 kõige enam alla-reguleeritud miRNA kolde stroomarakkudes. *In vitro* transfektsiooni katsete tulemusena tuvastasime, et miR-139-5p reguleerib *HOXA10* geeni ning miR-375 sihtmärgiks on *EDN1* geen.

Järeldused

1. Menstruaaltsükli jooksul naise kehas toimuvad hormonaalsed muutused ei mõjuta oluliselt vereplasmas ringlevate miRNA-de tasemeid, seega sobivad need molekulid mitte-invasiivsete biomarkeritena kasutamiseks ka reproduktiiveas naistel. Siiski tuleb arvesse võtta individuaalset erinevust rakuvabade miRNA-de tasemetes.
2. miR-200a, miR-200b ja miR-141 tasemed plasmas erinevad endometrioosiga patsientidel ja kontrollidel, kuid on tugevalt mõjutatud ka vereproovi võtmise ajast. Vähene spetsiifilisus ja sõltuvus haigusega mitteseotud teguritest viitavad nii meie kui ka teiste hiljuti teostatud sarnaste uuringute tulemuste põhjal sellele, et plasma miRNA-de potentsiaal endometrioosi biomarkeritena on kliiniliseks kasutamiseks ebapiisav.
3. Stroomarakkudes erinevalt ekspresseeritud mRNA ja miRNA integreeritud analüüsi tulemuste põhjal võime välja pakkuda kaks uut võimalikku endometrioosi patogeneesis olulist protsessi. *HOXA10* geeni on varasemalt seostatud endomeetriumi retseptiivsuse regulatsiooniga ning endometrioosiga naiste endomeetriumis on *HOXA10* tase madalam. Sellest lähtuvalt võib oletada, et miR-139-5p ekspressiooni ebanormaalne tase kolde rakkudes viib *HOXA10* taseme languseni, mis omakorda võib olla seotud endometrioosiga kaasneva viljatuse tekkes. miR-375 madala tasemega kolde stroomarakkudes kaasneb *EDN1* kõrgem ekspressioon. *END1* kõrgemat taset on varasemalt seostatud endometrioosi poolt põhjustatud valuga ning on näidatud antud geeni toimet mesenhümaalsetele tüvirakkudele – rakupopulatsioonile, mis oletatavalt mängib olulist rolli endometrioosi tekkel ja püsimajäämisel. Väljapakutud molekulaarsete mehhanismide tõendamiseks on siiski vaja täiendavaid funktsionaalseid uuringuid.

Kokkuvõtteks võib öelda, et endometrioosiga kaasneb olulisi molekulaarseid muutuseid, kuid vaja on veel palju edasist uurimistööd, et leida võimalus nende teadmiste rakendamiseks endometrioosiga naiste elukvaliteedi parandamiseks läbi uute diagnostiliste ja ravivõimaluste.

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