DISSERTATIONES
MEDICINAE
UNIVERSITATIS
TARTUENSIS
244

MERLI SAARE

Molecular Profiling of Endometriotic Lesions and Endometria of Endometriosis Patients

DISSERTATIONES MEDICINAE UNIVERSITATIS TARTUENSIS
244

DISSERTATIONES MEDICINAE UNIVERSITATIS TARTUENSIS
244

MERLI SAARE

Molecular Profiling of Endometriotic Lesions and Endometria of Endometriosis Patients

UNIVERSITY OF TARTU
Press

Institute of Biomedicine and Translational Medicine, University of Tartu, Estonia

Dissertation is accepted for the commencement of the degree of Doctor of Philosophy in Medicine on 16 March, 2016 by the Council of the Faculty of Medicine, University of Tartu, Estonia.

Supervisor: Professor Andres Salumets, PhD
Department of Obstetrics and Gynaecology / Institute of Biomedicine and Translational Medicine, University of Tartu, Estonia

Senior Researcher Maire Peters, PhD
Department of Obstetrics and Gynaecology, University of Tartu, Estonia

Professor Helle Karro, MD, PhD
Department of Obstetrics and Gynaecology, University of Tartu, Estonia

Reviewers: Associate Professor Kristiina Rull, MD, PhD
Department of Obstetrics and Gynaecology, University of Tartu, Estonia

Senior Researcher Lili Azin Milani, PhD
Estonian Genome Center, University of Tartu, Estonia

Opponent: Professor Martin Götte, PhD
Department of Gynecology and Obstetrics, University of Münster, Germany

Commencement: June 8, 2016

Publication of this dissertation has been granted by the University of Tartu, created under the auspices of European Social Fund.

ISSN 1024-395X
ISBN 978-9949-77-079-3 (print)
ISBN 978-9949-77-080-9 (pdf)

University of Tartu Press
www.tyk.ee

TABLE OF CONTENTS

LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following original articles:

I **Saare, M**; Sõritsa, D; Vaidla, K; Palta, P; Remm, M; Laan, M; Karro, H; Sõritsa, A; Salumets, A; D'Hooghe, T; Peters, M (2012). No evidence of somatic DNA copy number alterations in eutopic and ectopic endometrial tissue in endometriosis. Human Reproduction, 2012, 27(6), 1857–1864.

II **Saare, M**; Rekker, K; Laisk-Podar, T; Sõritsa, D; Roost, AM, Simm, J; Velthut-Meikas, A; Samuel, K; Metsalu, T; Karro, H; Sõritsa, A; Salumets, A; Peters, M (2014). High-throughput sequencing approach uncovers the miRNome of peritoneal endometriotic lesions and adjacent healthy tissues. PLoS One. 2014 Nov 11;9(11):e112630.

III **Saare, M**; Modhukur, V; Suhorutshenko, M; Rajashekar, B; Rekker, K; Sõritsa, D; Karro, H; Soplepmann, P; Sõritsa, A; Lindgren, CM; Rahmioglu, N; Drong, A; Becker, CM; Zondervan, KT; Salumets, A; Peters, M (2016). The influence of menstrual cycle and endometriosis on endometrial methylome. Clin Epigenetics. 2016 Jan 12;8:2.

Journal articles are reprinted with the permission from copyright holders.

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 the study design and data analysis, performing the experiments, writing the manuscript.

ABBREVIATIONS

AUC	–	area under the curve
BMI	–	body mass index
(a)CGH	–	(array-based) comparative genomic hybridization
CNV	–	copy number variation
CpGs	–	C-phosphate-G-sites
DIE	–	deep infiltrating endometriosis
DMR	–	differentially methylated region
EMT	–	epithelial to mesenchymal transition
ES	–	early-secretory phase
FC	–	fold change
FDR	–	false discovery rate
GWAS	–	genome-wide association study
GO	–	gene ontology
HE	–	hematoxylin and eosin
IHC	–	immunohistochemistry
KEGG	–	Kyoto Encyclopedia of Genes and Genomes
LCM	–	laser capture microdissection
lncRNA	–	long non-coding RNA
LS	–	late-secretory phase
M	–	menstrual phase
MDS plot	–	multidimensional scaling plot
MET	–	mesenchymal to epithelial transition
miRNome	–	the full spectrum of miRNAs expressed in a specific genome
MS	–	mid-secretory phase
miRNA	–	microRNA
PCA	–	principal component analysis
P	–	proliferative phase
SCNA	–	somatic copy number alteration
SD	–	standard deviation
SEM	–	standard error of the mean
SNP	–	single nucleotide polymorphism
SUP	–	superficial endometriosis
WGA	–	whole genome amplification
ZEB1	–	Zinc Finger E-Box Binding Homeobox 1
ZEB2	–	Zinc Finger E-Box Binding Homeobox 2
CSF	–	Clinical Score Formula

1. INTRODUCTION

Endometriosis is a frequent chronic gynaecological disease affecting not only women's health but also their life quality. Although endometriosis has been extensively studied for decades, the pathogenesis of the disease has remained enigmatic and the pathophysiology behind the development of endometriotic lesions is still largely unknown. Besides unknown pathogenesis, the diagnosis of endometriosis still depends on the findings from laparoscopic surgery as there are no non-invasive biomarkers with acceptable sensitivity and specificity enabling correct diagnostics. The search for potential biomarkers relies on the results of basic science research and thus, the molecular studies in endometriotic lesions and endometria are fundamentally important to understand the disease pathogenesis and to find new biomarkers for diagnostic purposes.

Why endometriosis develops and what are the molecular events triggering the implantation of endometrial cells into the ectopic locations, are the main questions any endometriosis researcher is faced with. The involvement of genetic factors in disease pathogenesis has been proposed and extensive effort has been made to reveal genetic background of the disease and to find possible molecular alterations responsible for disease development. Although the results of candidate gene association studies have not been very successful, the fast development of advanced and high-throughput technologies has opened new possibilities to get deeper insight into genetic, transcriptomic and epigenetic architecture of endometriotic lesions and endometria of endometriosis patients. To date, already more than 50 comprehensive profiling studies searching for molecular alterations in endometriotic lesions and eutopic endometria of endometriosis patients have been conducted and hundreds of potential markers have been proposed. However, the concordance between the results of these studies has remained moderate and new concerns have emerged. The concern about proper study design has been lately raised and the guidelines helping to harmonize endometriosis studies have been established. The relevance of study design in endometriosis research cannot be underestimated and all interfering factors (e.g. the composition and location of examined tissues and the effect of menstrual cycle on molecular processes) should be considered if conducting molecular profiling studies.

The general aim of the current thesis was to use comprehensive molecular tools to assess genetic, transcriptomic and epigenetic profiles of eu- and ectopic endometrial tissues and thereby to discover molecular events accompanying endometriosis development.

2. REVIEW OF LITERATURE

2.1. Endometriosis epidemiology

Endometriosis is a common estrogen-dependent benign gynaecological disease affecting 5–10% of women of reproductive age and up to 50% of women with pelvic pain and infertility (Giudice, 2010). The exact prevalence of endometriosis in Estonia is unknown but on average 300 women (0.1% of reproductive-aged women) get the diagnosis of endometriosis every year (data from Estonian Health Insurance Fund and Statistics Estonia, January 2016). Endometriosis is characterized by the growth of functional endometrial tissue outside the uterine cavity. The cells growing in ectopic locations are under the influence of female hormones and follow the normal menstrual cycle, similarly to endometrial cells inside the uterus. During menstruation, the bleeding from desquamated ectopic lesions causes inflammatory processes inside the peritoneal cavity leading to the formation of adhesions and causing pain and infertility. The most common symptom of endometriosis is chronic or cyclic pain (pelvic pain, pain during menstruation and ovulation, painful sexual intercourse) and thus endometriosis has a huge impact on the quality of life of these women (Dunselman et al., 2014). In addition to pain symptoms, endometriosis has also a major impact on women's fertility, as it affects the ovarian reserve, causes poor oocyte and embryo quality and low implantation rates; however, the mechanisms behind endometriosis-associated infertility are still poorly understood (reviewed in Macer et al., 2012).

The symptoms of endometriosis are nonspecific and it is proposed that the disease is underdiagnosed with an average delay of 7 years between the onset of the symptoms and the correct diagnosis (Rogers et al., 2013). The delay of the diagnosis can be explained by the fact that despite of extensive research, there are still no non-invasive diagnostic markers for endometriosis and according to the guidelines of European Society of Human Reproduction and Embryology (Dunselman et al., 2014) the gold standard for accurate diagnosis of endometriosis is still laparoscopy together with histological verification of endometrial glands and/or stromal cells of biopsied samples. The non-invasive imaging technologies like magnetic resonance imaging and transvaginal ultrasound can be used for diagnosing large endometriomas and deep endometriotic nodules, though for accurate diagnosis (to determine severity of the disease, presence of adhesions, small endometriomas and superficial lesions) and for surgical treatment, laparoscopic surgery is still needed. Due to the invasive nature of endometriosis diagnostics, robust and specific non-invasive biomarkers with acceptable sensitivity and specificity, and detectable preferably from easily assessable sources like blood and body fluids, are needed. Although endometriosis biomarkers have been sought from peripheral blood (whole blood, plasma, serum), menstrual blood, peritoneal fluid and urine samples and more than 100 markers, among them annexin V, CA-125, glycodelin, glycoproteins, inflammatory and non-inflammatory cytokines, angiogenic and growth

factors have been assessed, the results of these studies have been inconsistent and have not provided diagnostic tests with sufficient predictive power (May et al., 2010; Vodolazkaia et al., 2012).

The severity of endometriosis is classified into four stages (I-minimal, II-mild, III-moderate, and IV-severe) depending on the location, extent and depth of lesions, presence and extent of adhesions and endometriomas (ASRM, 1997). Women with minimal-mild (stage I–II) disease have superficial lesions and mild adhesions, women with moderate-severe endometriosis (stages III–IV) have usually superficial/deep infiltrating endometriosis (DIE) or endometriomas and severe adhesions. Curiously, the symptoms of endometriosis and severity of the disease correlate poorly with fertility outcome, severity of the pain or characteristics of lesions (type, size and location), making the diagnosis and research to reveal disease pathogenesis even more complicated.

Although epidemiologic studies have found several risk factors for endometriosis such as menstrual cycle characteristics (early age at menarche, short menstrual cycles and heavy bleeding), taller height, decreased body mass index (BMI) and low number of pregnancies (reviewed in Cramer et al., 2002) the questions "why endometriosis develops, how endometrial cells reach to ectopic sites and how to find biomarkers for non-invasive diagnostics" have remained the main initiators for many basic science studies that will be summarized in the following sections.

2.2. Endometriotic lesions morphology and histology

As the location and general appearance of endometriotic lesions (such as colour, size and morphology) are highly variable between patients (Hsu et al., 2010), histological evaluation of biopsied lesions is needed to confirm the diagnosis of endometriosis and to facilitate optimal treatment. The most common locations of lesions are on the surface of pelvic cavity organs (ovaries, uterus and fallopian tubes), ligaments (uterosacral ligaments, broad and round ligaments), recto-uterine pouch and ovarian fossa. Extra-pelvic locations of lesions are rare but in some cases lesions can be found in upper abdomen, diaphragm but also on operation scars, urinary tract, bowel, appendix and rectum. Endometriotic lesions (Figure 1) can be divided into three different subtypes: firstly, superficial peritoneal lesions which may be black, dark-brown, blue "powder-burn", red, clear lesions or white areas of fibrosis, located mainly in pelvic peritoneal tissue and organs. Secondly, endometriomas that are ovarian cysts lined by endometrial tissue and filled with old dark-brown blood; and thirdly, DIE, in which case the endometrial cells penetrate > 5 mm into the adjacent fibromuscular tissue (reviewed in Hsu et al., 2010). It has been proposed that colour of the endometriotic lesions changes over time, starting from red, progressing to black and finally reaching the fibrotic white lesions (Nisolle et al., 1997). Different subtypes of endometriotic lesions can be found in combination, or just a single subtype can be present. To date, it is unclear whether different lesion subtypes share common pathologic mechanisms (Nisolle et al., 1997).

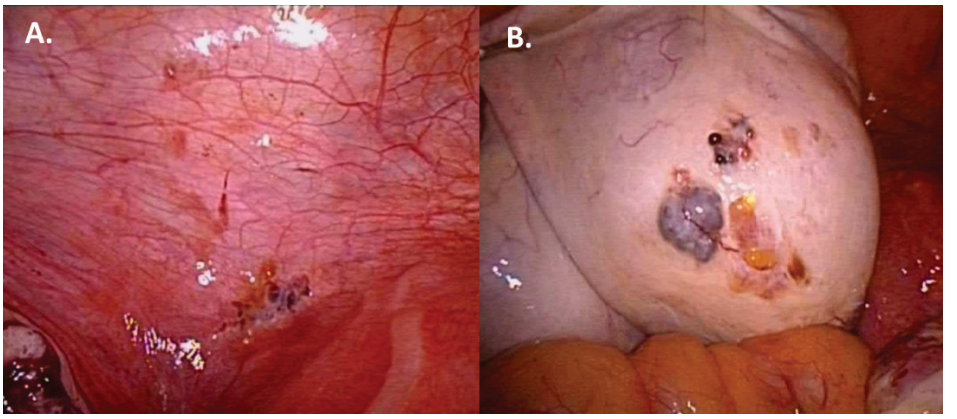

Figure 1. Photographs of endometriotic lesions. **A.** Peritoneal superficial endometriotic lesions on the *Ligamentum sacrouterina*. **B.** Endometriotic lesions on the surface of the ovarian mucinous cystadenoma. Photographs made by Dr. Pille Soplepmann (Tartu University Hospital, Estonia).

The histological diagnosis of endometriosis requires the presence of at least two histological features in the lesion: either endometrial epithelial cells, endometrial stromal cells surrounding the glandular epithelial cells or hemosiderin-laden macrophages (Anaf et al., 2000) (Figure 2). During the routine histological assessment, several tissue sections from different parts of the biopsy are examined but identification of specific cells may still be complicated as many lesions with "typical" macroscopic appearance cannot be confirmed at the microscopic level (Moen et al., 1992; Walter et al., 2001). Although histological evaluation of lesions is routinely used in everyday practice, as many as 30–50% of surgical specimens removed during laparoscopy are not confirmed by histological assessments (Moen et al., 1992; Stratton et al., 2003; Stegmann et al., 2008; Fernando et al., 2013). In endometriomas, the histopathological validation is even more variable ranging from 0% (Fayez et al., 1991) to 100% (Muzii et al., 2007). The possible reasons why histological assessment of the lesions may fail are inadequate sample collection during the laparoscopy, presence of lesion-like tissue structures in abdominal cavity, very small size of lesions or because samples may lack endometriotic glands, and sparse stromal cells may be hidden in surrounding tissue (Kennedy et al., 2005). The most common histopathologic findings of lesion-like structures include fibrosis, hemorrhagic changes, inflammatory changes and normal peritoneal tissue (Wanyonyi et al., 2011) and for endometrioma-like cysts, ovarian tissue, non-diagnostic cysts and corpus luteum or albicans cysts are the usual findings (reviewed in Muzii et al., 2007).

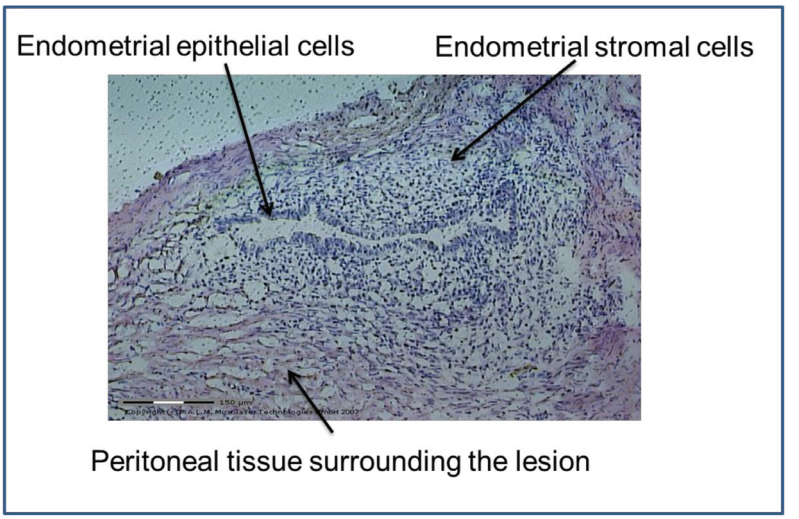

Figure 2. Photomicrograph of peritoneal endometriotic lesion. Section of *Ligamentum sacrouterina*, 10 × magnification, 10 µm section stained with hematoxylin and eosin. Arrows indicate endometrial stromal and epithelial cells and adjacent healthy peritoneal tissue.

2.3. Pathogenesis of endometriosis

Despite of the extensive studies, the pathogenesis of the endometriosis has remained a mystery and there are several theories as to how endometrial cells reach to ectopic sites and which factors trigger the ectopic growth of the cells (Figure 3). The most popular Sampson's retrograde menstruation theory postulates that endometrial cells are transported together with menstrual blood into the peritoneal cavity (Sampson, 1927). The coelomic metaplasia theory says that endometriosis arises from specialised cells from mesothelial lining of the visceral and abdominal peritoneal tissue, while the lympho-vascular metastasis theory hypothesizes that endometrial cells spread with lymphatic or hematologic flow. The theory of embryonic Müllerian rests proposes that cells of the Wolffian or Müllerian ducts maintain capacity to develop into lesions, whereas stem cell theory claims that extrauterine stem cells may differentiate into endometriotic tissue. The theory of peritoneal origin postulates that alterations in peritoneal mesothelial cells facilitate the binding and invasion of cells in extracellular matrix (Sasson et al., 2008; Burney et al., 2012; Young et al., 2013). Most recent theories of fetal origin of endometriosis and tissue post-traumatic theory suggest that endometriosis may develop during the early embryogenesis in response to molecular alterations that lead to disruption of the fine-tuning mechanisms responsible for the correct development of the female genital system (Signorile et al., 2010a) or small microtrauma events could be the initial events triggering the disease development (Canis et al., 2016). Also, the involvement of environmental factors like dioxin, endocrine system

modifications, oxidative stress and inflammation, inefficient clearance from menstrual debris in peritoneal cavity caused by immune or inflammatory deficiency, alterations in cell fate and apoptosis systems and genetic/epigenetic factors or a combination of these mechanisms in disease pathogenesis have been proposed (reviewed in Burney et al., 2012).

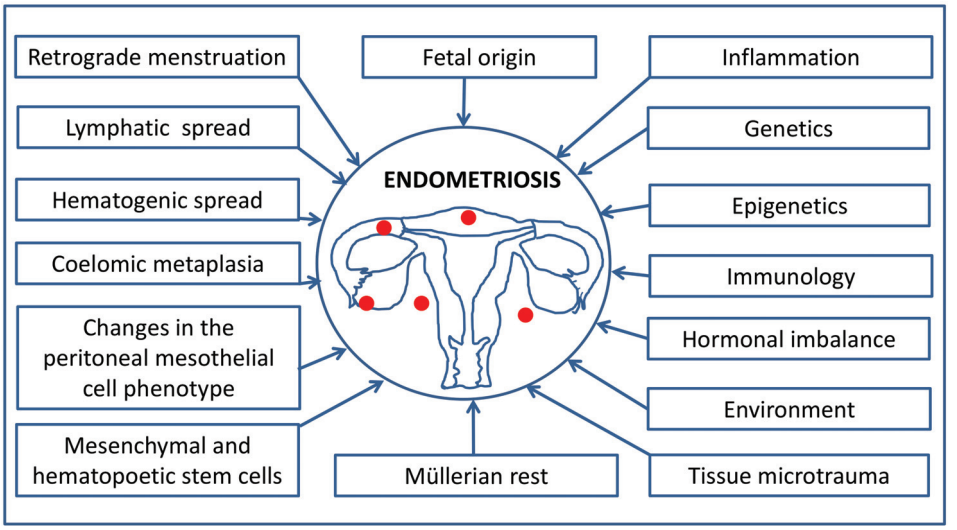

Figure 3. The main theories of endometriosis development.

Although there are many theories, no single theory can explain all aspects of the disease development. The most widely accepted Sampson's retrograde menstruation theory is supported by multiple lines of evidence, such as the presence of viable endometrial cells in menstrual and peritoneal fluid (Koninckx et al., 1998), development of endometriotic lesions in non-human primates after inoculation of endometrial tissue into peritoneal cavity (D'Hooghe et al., 1991) and higher frequency of endometriosis in women with congenital abnormalities causing obstruction of outflow (Sanfilippo et al., 1986). However, retrograde menstruation is a universal phenomenon among healthy women with patent fallopian tubes and therefore this theory does not explain why endometriosis is present in only 5–10% of women. Why endometrial cells, irrespective of the mechanisms how they reach to the peritoneal cavity, are able to attach, invade and form ectopic endometrial lesions in some women is currently unknown, but the endometrial origin of endometriotic cells is widely accepted. Furthermore, it has been proposed that genomic, transcriptomic and epigenomic alterations in endometrial cells of some women could be responsible for the elevated ability of these cells to attach and grow in ectopic locations.

2.4. Molecular profiling studies in endometriotic lesions and endometria

The advancements in genome-wide microarray and sequencing technologies started the molecular profiling (omics) era in endometriosis studies that has revolutionized our understanding about the disease background. Since 1999, around 50 publications using high throughput technologies and focusing on genomics, epigenomics and transcriptomics of endometriotic lesions and endometrium have been published (Figure 4).

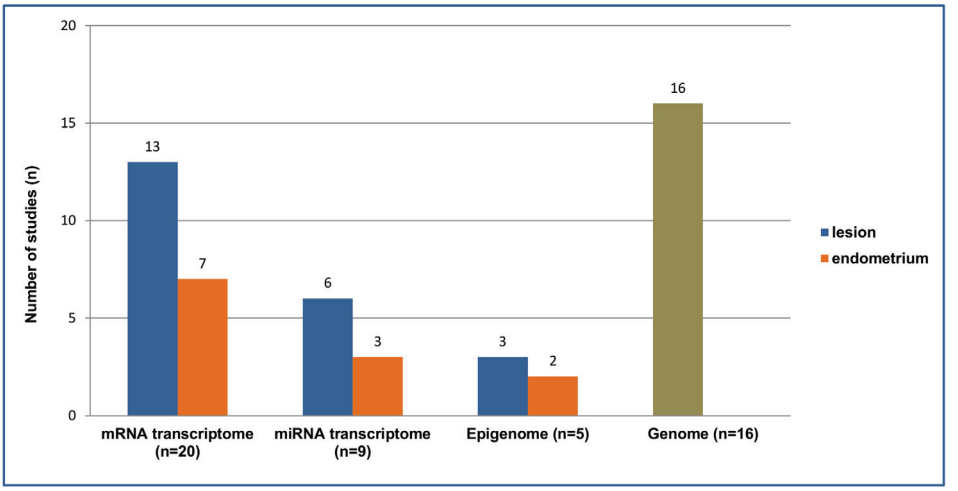

Figure 4. Molecular profiling studies in endometriosis. X-axis denotes the type of 'omics' analysed, while Y-axis indicates the number of studies. The numbers above the bars indicate the numbers of the studies. "Lesion" indicates studies using either lesions or lesions together with endometrium, while "endometrium" indicates those studies using only endometria. Literature search was performed in PubMed, including studies published up to January 2016. Only publications in English were considered. The keyword 'endometriosis' was one-by-one searched in combination with terms: 'endometrium', 'miRNA+microarray', 'sequencing', 'microarray', 'gene expression+microarray', 'exome sequencing', 'GWAS', 'CNV', 'genomics', 'DNA methylation', 'DNA alterations'. Some of the eligible studies were identified using the reference lists of appropriate review articles. Epigenome studies include only papers describing DNA methylation.

Genomics is defined herein as variability in DNA sequence at the genome level, epigenomics as epigenetic modifications of DNA, and transcriptomics as variability in composition and abundance of mRNA and microRNA (miRNA) levels (Altmae et al., 2014). The major advantage of using molecular profiling when studying complex diseases with unknown pathogenesis such as endometriosis, is that the data can be collected without existing hypotheses and a primary research question is not always needed (first experiment-then-hypothesize approach) (Ozdemir et al., 2009). Although the amount of molecular

profiling studies in endometriosis is still moderate, the number of studies is constantly growing because of the accessibility and affordability of new methods. A detailed review of 'omics'-level studies in endometriosis is given in the following chapters.

2.4.1. Genomics of endometriosis

2.4.1.1. Single nucleotide polymorphisms in endometriosis

Endometriosis is a polygenic complex disease, which means its pathogenesis is affected by many different gene variants together with environmental influences. Comprehensive genome-wide association studies (GWAS) comprising thousands of individuals and including hundreds of thousands single nucleotide polymorphisms (SNPs) together with further replication studies (Adachi et al., 2010; Uno et al., 2010; Painter et al., 2011; Nyholt et al., 2012; Albertsen et al., 2013; Pagliardini et al., 2013; Sundqvist et al., 2013; Sapkota et al., 2015) have been performed to elucidate the genetic predisposition to endometriosis. A recent meta-analysis included more than 11,506 patients and 32,678 controls and found that six loci were genome-wide significant, including rs12700667 on 7p15.2, rs7521902 near *WNT4*, rs10859871 near *VEZT*, rs1537377 near *CDKN2B-AS1*, rs7739264 near *ID4* and rs13394619 in *GREB1* (Table 1) (Rahmioglu et al., 2014b).

Table 1. Results of the GWAS metaanalysis (Rahmigiolu et al. 2014)

SNP	OR (95% CI)*	P value	Nearest gene
rs7521902	1.18 (1.13–1.23)	1.8×10^{-15}	*WNT4*
rs12700667	1.13 (1.08–1.17)	1.9×10^{-9}	intergenic
rs1537377	1.12 (1.08–1.17)	1.0×10^{-8}	*CDKN2B-AS1*
rs10859871	1.18 (1.13–1.22)	4.8×10^{-15}	*VEZT*
rs7739264	1.11 (1.08–1.15)	1.8×10^{-10}	*ID4*
rs13394619	1.13 (1.07–1.20)	4.5×10^{-8}	*GREB1*

*OR, odds ratio, CI confidence interval

Although GWAS have provided valuable information about SNPs and reported novel candidate genes as well as genome regions, the effect sizes for the associated variants are quite moderate (odds ratios between 1.0–1.2). It is very likely that instead of common variants the rarer variants (minor allele frequency of <0.05) not captured by the GWAS genotyping arrays used in endometriosis studies thus far, could contribute to the risk of the disease (Visscher et al., 2012).

2.4.1.2. Inherited DNA copy number variations in endometriosis

Besides finding the involved gene variants and genes, genomic studies enable searching for inherited DNA copy number variations (CNVs). High-resolution SNP arrays, together with comparative genomic hybridization (CGH) and array-CGH (aCGH) provide the opportunity to assess inherited and sporadic CNVs that may potentially contribute to development of many diseases, including endometriosis. Inherited CNVs, present in all cells of the human body, are relative small deletions or duplications of genomic segments that change the copy-number status of the specific genomic regions covering up to 13% of human genome (Stankiewicz et al., 2010). The loss or gain of specific genomic regions can lead to alterations in gene expression and it has been proposed that approximately 18% of gene expression variability comes from the presence of CNVs (Stranger et al., 2007). To date, there are more than three million CNVs (range from 100 bp to several Mb in size) reported in the database of genomic variants (DGV). Although many disease-related CNVs have been described (in association with hypertension, rheumatoid arthritis, type 1 diabetes, autism, breast cancer, obesity, Alzheimer disease etc.), large population-based CNV studies have found substantial variability in CNV distribution also in healthy individuals (Sebat et al., 2004; Pinto et al., 2007; Teo et al., 2011; Li et al., 2013).

Only one large-scale study for genomic CNVs from saliva samples in endometriosis has been conducted (Chettier et al., 2014). This case-control study compared 2,126 surgically confirmed endometriosis cases to 17,974 controls and found that there were no significant differences neither in CNV counts, nor in proportion of large CNVs or gene-based CNVs between controls and patients (Chettier et al., 2014). Although this study failed to find any large CNVs that contribute to the pathogenesis of endometriosis, the analysis revealed 22 rare CNVs that were detected in 6.9% of affected women compared to 2.1% of the general population. Three out of 22 CNVs passed a genome-wide P-value threshold, namely a deletion at *SGCZ* on 8p22, a deletion in *MALRD1* on 10p12.31, and a deletion at 11q14.1 (Chettier et al., 2014). Although little is known about the function of *MALRD1* and the CNV at 11q14.1 located in gene desert, the deletion of *SGCZ* (member of the sarcoglycan gene-family) that is a component of the vascular smooth muscle sarcoglycan complex could be involved in endometriosis pathogenesis as smooth muscle structures have been found in endometriotic lesions (Barcena de Arellano et al., 2014). As these CNVs have not been previously associated with endometriosis, further replication studies in different populations are needed to confirm the findings.

2.4.1.3. *De novo* somatic DNA alterations in endometriosis

In addition to inherited CNVs that are present in all cells of the human body, the non-inherited alterations in somatic cells (somatic copy number alterations, SCNAs) that arise spontaneously, have gained attention in endometriosis

research. The main hypothesis encouraging SCNA studies has been based on the idea that endometriosis is a tumor-like disease caused by *de novo* somatic DNA aberrations (Figure 5) that are frequent in malignant neoplasms (Beroukhim et al., 2010). How and where these alterations occur is still unclear but the primary source of SCNAs in endometriosis could be either the eutopic endometrium from which menstrual blood containing endometrial cells with changed properties refluxes through the fallopian tubes to peritoneal cavity and form lesions or somatic alterations may arise in normal endometrial cells at ectopic locations in response to abnormal peritoneal environment.

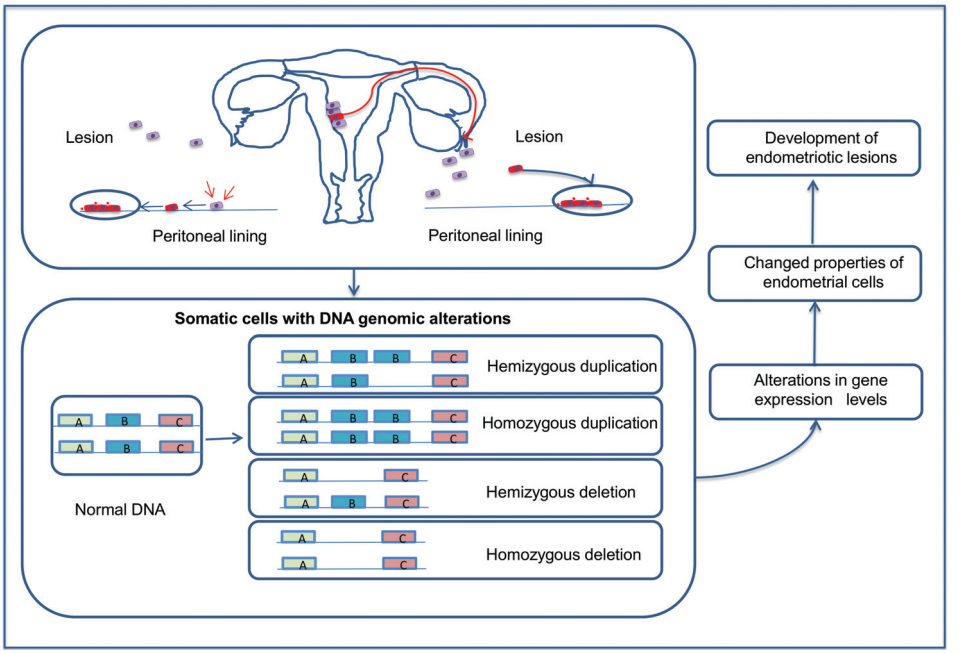

Figure 5. The schematic representation of putative mechanisms of endometriotic lesion development through SCNAs. Genomic alterations may occur already in the eutopic endometrium (denoted in the right side of the upper panel) or arise in response to peritoneal environmental conditions from normal endometrial cells that are refluxed during the menstruation into the abdominal cavity (denoted in the left side of the upper panel). Lower panel: normal DNA has two copies of each specific genomic region, homozygous duplication indicates the presence of 4 copies, hemizygous duplication 3 copies, hemizygous deletion 1 copy and homozygous deletion indicates a loss of specific genomic region.

Different methods have been used (e.g. traditional methods like karyotype analysis, fluorescence *in situ* hybridisation, microsatellite analysis, and genome-wide methods like CGH, aCGH and exome sequencing) to identify SCNAs in eutopic or ectopic endometrial tissues of endometriosis patients (Gogusev et al., 1999; Guo et al., 2004; Wu et al., 2006b; Zafrakas et al., 2008; Silveira et al., 2012; Yang et al., 2013) (Table 2). However, the results of these studies have

not provided a clear consensus about the presence of somatic alterations either in endometriotic lesions or eutopic endometria. Some of the studies have reported the presence of chromosomal alterations in lesions, more frequently gains in chromosomes 1p, 3p, 6q, 17q, and Xq and losses in chromosomes 1p, 5p, 6q (Gogusev et al., 1999; Wu et al., 2006b; Veiga-Castelli et al., 2010; Silveira et al., 2012; Yang et al., 2013), while the study by Zafrakas et al. (2008) found no chromosomal aberrations in ectopic or eutopic endometrium (Zafrakas et al., 2008). Guo et al. (2004) found several genomic alterations also in eutopic endometria of endometriosis patients and hypothesised that these alterations could be the proximate cause of endometriosis (Guo et al., 2004). Recently, the first study implementing whole-exome sequencing to search somatic alterations in endometriosis was conducted by Li et al. (2014) who sequenced the exomes of blood DNA and laser capture microdissection (LCM)-harvested endometrial cells from eutopic and ectopic endometria of 16 endometriosis patients and eutopic endometria of 5 healthy women (Li et al., 2014). A very interesting finding of this study was that DNA originating from healthy endometria contains thousands of somatic mutations that are absent in blood DNA, indicating the presence of somatic mutations also in endometria of healthy women. The overall somatic mutation spectrum in endometria of women with and without endometriosis was very similar; however, endometriotic cells from patients had a unique fingerprint and a number of mutated genes were related to pathways involved in adhesion processes, chromatin modification, cell cycle, DNA repair and regulation of apoptosis (Li et al., 2014). The authors concluded that most of the mutations are already present in the cells of eutopic endometrium in endometriosis patients and nearly all of these mutations are probably benign and irrelevant for pathogenesis (Li et al., 2014).

Based on the inconclusive results of aforementioned studies it is difficult to draw any definite conclusion about the presence and potential role of SCNAs in endometriosis pathogenesis and therefore further studies are needed.

Table 2. Genome-wide somatic DNA alteration studies in endometriosis

Detection method	Studied tissue samples (n)	Frequent DNA somatic alterations	Reference
Exome sequencing	Matched blood (16), LCM harvested cells: lesion (16) and endometrium (16) Healthy endometria (5)	Mutations in eutopic endometria (n=724) and ectopic endometrium (n=465)	(Li et al., 2014)
aCGH	Lesion (11) Endometrium (11)	Gains: 20q13.33	(Yang et al., 2013)
High resolution-CGH	LCM harvested stromal and epithelial cells: lesion (20) and endometrium (3)	Stromal cells- gains: 1p, 2q, 3p, 4q, 5, 6, 8q,9p, 10p, 12 and X and losses: 1p, 3q, 4q, 5p, 5q, 8p, 13q, 14q, 19p and X. Epithelial cells- gain: 4p, losses: 6p, Xq	(Silveira et al., 2012)
CGH	Lesion (n=10) Endometrium (n=10)	Gains:11q, 17p, 17q, 19p	(Veiga-Castelli et al., 2010)
aCGH	Lesion (n=10)	–	(Zafrakas et al., 2008)
aCGH	Lesion (n=5) Endometrium (n=5) Healthy endometria (4)	Gains: 1p, 6p, 6q, 11p, Xq, losses: 1p, 5p, 6q, 16q	(Wu et al., 2006b)
aCGH	LCM harvested stromal and epithelial cells Endometrium (n=5) LCM harvested stromal and epithelial cells Healthy endometrium (n=4)	Gains: 3p, 10q, 13q, losses:1p, 3p, 4p, 22q	(Guo et al., 2004)
CGH	Lesion (n=18)	Gains: 1q, 6q, 7q, 17q losses: 1p, 5p, 6q, 7p, 22q, 9q, 16q, 17q	(Gogusev et al., 1999)

LCM – laser capture microdissection, (a)CGH – (array-based) comparative genomic hybridization

2.4.2. Transcriptome studies in endometriosis

Since 2002, around 20 mRNA transcriptome studies applying microarray technology have been carried out to reveal the gene expression profile specific to eutopic endometria and endometriotic lesions of endometriosis patients. Studies focusing only on eutopic endometria of patients and controls (Kao et al., 2003; Absenger et al., 2004; Matsuzaki et al., 2005; Burney et al., 2007; Sherwin et al., 2008; Aghajanova et al., 2011; Fassbender et al., 2012; Tamaresis et al., 2014) have not provided clear evidence about the importance of transcriptomic changes in eutopic endometria to disease pathogenesis. Although variable amounts of differentially expressed genes have been reported, with little concordance between the studies (Figure 6A), it has been proposed that the influence of menstrual cycle phases has a more pronounced effect on endometrial transcriptome than the presence of endometriosis (Burney et al., 2007; Aghajanova et al., 2011; Fassbender et al., 2012).

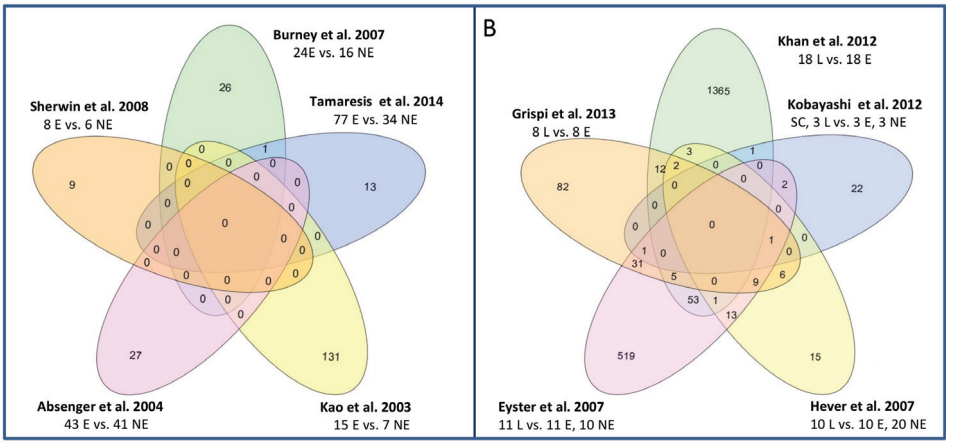

Figure 6. Venn diagrams describing the overlap between the results from different transcriptome studies. **A**. transcriptome studies in eutopic endometria and **B.** transcriptome studies where endometriotic lesions were compared to eutopic endometria. The input for Venn diagrams is based on the literature review and only studies presenting differentially expressed gene lists were included. In some studies only the top lists (Burney et al., 2007; Hever et al., 2007; Kobayashi et al., 2012) or partial lists (Absenger et al., 2004; Eyster et al., 2007; Tamaresis et al., 2014) of genes were eligible for analysis. The numbers of studied tissue samples are shown on the figure together with the respective references. E, endometriosis endometrium; NE, healthy or non-endometriosis endometrium; L, endometriotic lesion; SC, primary endometrial stromal cell cultures.

Transcriptome studies in lesions have found a remarkable number of genes with altered expression in ectopic endometrial tissue while compared to eutopic endometria (Lebovic et al., 2002; Arimoto et al., 2003; Konno et al., 2003; Matsuzaki et al., 2004; Hu et al., 2006; Wu et al., 2006a; Eyster et al., 2007; Khan et al., 2012; Crispi et al., 2013; Sun et al., 2014). Similarly to the endo-

metrial tissue studies, the comparison of available gene lists (Eyster et al., 2007; Hever et al., 2007; Khan et al., 2012; Kobayashi et al., 2012; Crispi et al., 2013) showed that the overlap between these studies was relatively small (Figure 6B) and only a small subset of genes (e.g. *AEBP1*, *CXCL2*, *C3* and *C4A*) was found in more than three studies, while no differentially expressed genes common to all studies were reported.

Beside mRNAs, the first studies focusing on long noncoding RNAs (lncRNAs) have been conducted (Sun et al., 2014; Wang et al., 2015). lncRNAs are thought to function as regulators of gene expression at almost every stage, from targeting epigenetic modification to modulation of mRNA stability and translation (Mercer et al., 2013). Authors proposed that many found dysregulated lncRNAs may participate in biological pathways related to chromosome condensation, nucleosome assembly, and protein–DNA complex assembly, which correspond well to the central role lncRNAs play in the epigenetic regulation of the genome (Sun et al., 2014). However, the true relevance of lncRNAs in pathogenesis of endometriosis needs to be elucidated in future studies.

It should be pointed out that to date no meta-analyses have been conducted concentrating on all endometriosis transcriptome data, probably because of the insufficient data presented in papers and also because of the poor availability of full datasets. Furthermore, the study design differences make the comparison of findings even more complicated. For example, lesions from different locations (peritoneal lesions/endometriomas) have been compared to healthy or matched endometrium, selection criteria for patients and controls have been different, used array types vary, and menstrual cycle phases of studied individuals are not reported or samples from different menstrual cycle phases have been used. Still, despite the low concordance between results, all transcriptome studies have found remarkable differences between lesions and eutopic endometria and therefore, future large scale studies with carefully planned study design are needed to reveal changes truly related to the disease pathogenesis.

2.4.3. miRNA studies in endometriosis

miRNAs are small (typically 22 nucleotides in size) non-coding regulatory RNA molecules, which have an important function in modulating the stability of specific mRNA targets. Changes in miRNA expression that affect target mRNA degradation and/or translation may cause alterations in the dynamic balance between miRNAs and their target mRNAs and thereby lead to changes in normal physiological status of the tissues. miRNAs have been extensively studied in association with different pathologic conditions, among them uterine and endometrial disorders such as uterine leiomyoma (Creighton et al., 2010), endometrial carcinoma (Banno et al., 2013) and endometriosis (reviewed in Gilabert-Estelles et al., 2012). miRNA studies in endometriosis can be divided into two groups: studies focusing on endometria of patients and controls, and

studies comparing miRNA expression profiles between endometriotic lesions and endometrium.

Some microarray-based miRNA studies describing differences between eutopic endometria of patients with endometriosis and healthy women have been performed (Burney et al., 2009; Laudanski et al., 2013; Braza-Boils et al., 2014; Shi et al., 2014; Laudanski et al., 2015). Burney et al. found differential expression of two miRNA families, miR-9 (miR-9, miR-9*) and miR-34 (miR-34b*, miR-34c-5p, miR-34c-3p) in patients with endometriosis (Burney et al., 2009). A subsequent study by Laudanski et al. reported two miRNAs (miR-483-5p and miR-629) that were down-regulated in eutopic endometrium of patients compared to controls (Laudanski et al., 2013). Authors suggested that lower expression of these miRNAs is a consequence of an early defect in the physiological activity of the proliferative endometrium, eventually resulting in the overgrowth of this tissue outside the uterus (Laudanski et al., 2013). Laudanski et al. expanded their previous work and reported the presence of 136 upregulated miRNAs in the eutopic endometrium of patients with advanced ovarian endometriosis compared with the eutopic endometrium (Laudanski et al., 2015). After validation of 11 miRNAs, only three: miR-5187-3p, miR-3152-5p, and miR-30d-5p, revealed borderline significance. Furthermore, authors were not able to detect the differential expression of miRNAs they had reported in their previous study (miR-483-5p and miR-629) (Laudanski et al., 2013). In the study by Braza-Boils et al. (2014) both eutopic endometria from patients and controls and endometriotic tissues were studied (Braza-Boils et al., 2014). The hierarchical clustering analysis showed clearly that the miRNA expression signatures of eutopic endometria of patients and controls were very similar and only five miRNAs were found to be differentially expressed (fold change, FC > 1.5) in eutopic endometria. Thirty six downregulated miRNAs in endometria of patients were also reported by Shi et al. (Shi et al., 2014). However, the comparison of all the results from aforementioned miRNA studies (Figure 7A) showed only a minute overlap, and only one miRNA (miR-9*) was reported to have altered expression in eutopic endometria of endometriosis patients in two studies. Is the small overlap a sign that miRNA expression levels are not affected in eutopic endometria of endometriosis patients or is it a reflection of differences in study design (array platforms/the effect of menstrual cycle/ selection of controls etc.), is not clear.

Genome-wide miRNA studies describing the full spectrum of miRNAs expressed in a specific genome (miRNome) of peritoneal lesions or endometriomas have found a number of miRNAs that could be associated with disease pathogenesis (Ohlsson Teague et al., 2009; Filigheddu et al., 2010; Hawkins et al., 2011; Braza-Boils et al., 2014; Shi et al., 2014). Similarly to endometrial studies, the concordance between endometriotic lesion studies is relatively small and no differentially expressed miRNAs can be found in all studies (Figure 7B). However, subsets of overlapping miRNAs such as miR-200b and miR-196b (found in four studies), miR-200a, miR-183, miR-375, miR-200c and

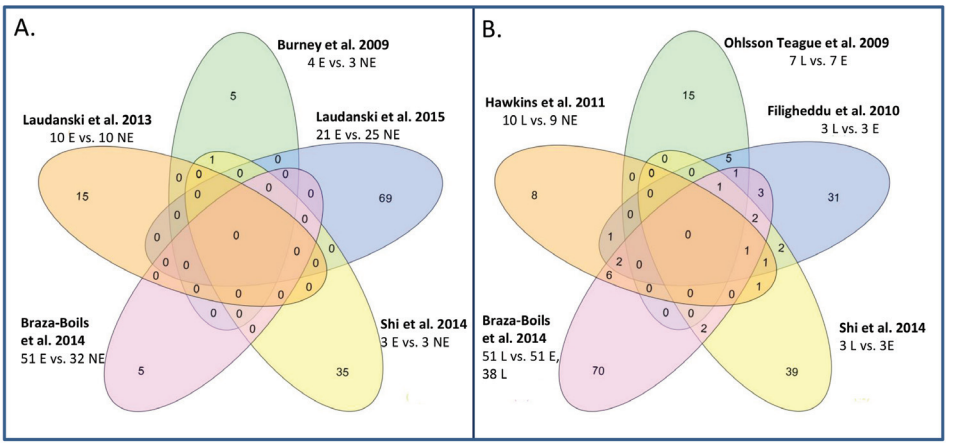

Figure 7. Venn diagrams describing the overlap between the results from different miRNA studies. **A**. miRNA studies in eutopic endometria and **B.** miRNA studies where endometriotic lesions were compared to eutopic endometria. miRNAs included into the analysis were with FC > 1.5 and p-value < 0.05. The input for Venn diagram is based on the literature review and only studies presenting differentially expressed miRNA lists were included. Only the top lists of miRNAs were provided in some studies (Burney et al., 2009; Laudanski et al., 2013). The numbers of studied tissue samples are shown on the figure together with the respective references. E, endometriosis endometrium; NE, healthy or non-endometriosis endometrium; L, endometriotic lesion.

miR-29c (found in three studies) and miR-381, miR-133b, miR-429, miR-10a, miR-34c-5p, miR-141, miR-449b, miR-509-3-5p, miR-1, miR-182, miR-376a, miR-203, miR-93, miR-25, miR-100, miR-20a miR-150 miR-145 miR-99a and miR-365 (found at least in two studies) have been repeatedly reported. Interestingly, the members of miR-200 family (miR-200a, miR-200b and miR-141) have been frequently reported to be differentially expressed between lesions and endometria. miR-200 family has been shown to target a complex network of important transcription regulators like ZEB1 and ZEB2 (E-box-binding transcription factors 1 and 2) which are transcriptional repressors for E-cadherin (Figure 8) (Park et al., 2008). The members of this complex regulate two essentially important biological processes – cell migration and epithelial-mesenchymal transition (EMT), which are supposed to be crucial events for the development of endometriosis (Matsuzaki et al., 2012). During the EMT process epithelial cells lose their specific features and acquire mesenchymal characteristics. It has been shown that these miRNAs are involved in maintaining the epithelial nature of the cells by downregulating the E-cadherin repressors ZEB1 and ZEB2 and this process is regulated by the double-negative regulatory ZEB/miR-200 feedback loop where ZEB1 and ZEB2 bind also to the E-box elements located at the promoter regions of the miR-200 family members (Bracken et al., 2008; Brabletz et al., 2010). There is strong evidence that overexpression of ZEB1/ZEB2 and the subsequent loss of E-cadherin expression lead to increased cell invasion and migration, loss of epithelial

integrity and thereby could initiate pathological processes (Korpal et al., 2008; Park et al., 2008; Romero-Perez et al., 2013).

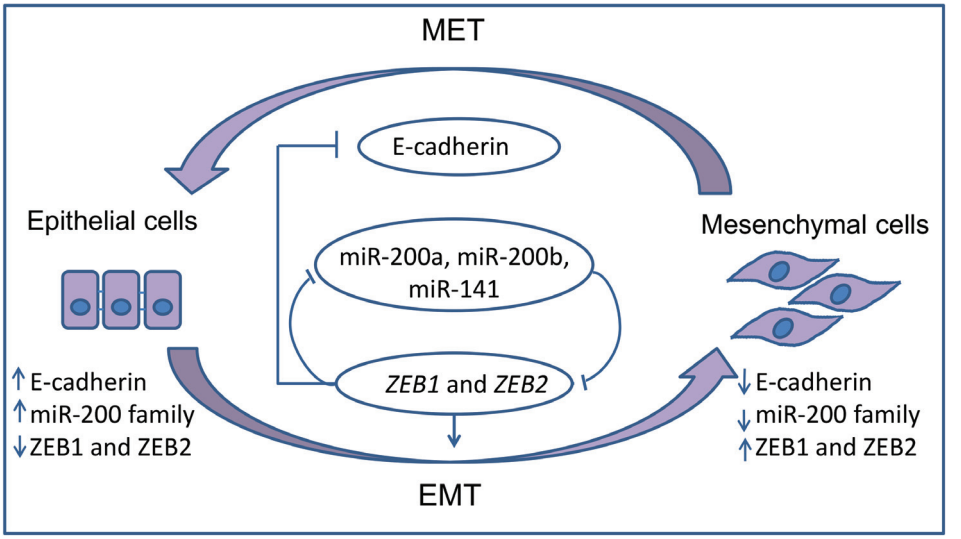

Figure 8. The schematic representation of the miR-200 family members' role in regulation of EMT and mesenchymal-epithelial transition (MET) processes. miR-200 family members regulate EMT directly by targeting the E-cadherin repressors ZEB1 and ZEB2 mRNA. ZEB1 and ZEB2 bind also to the promoter regions of the miR-200 family members, forming double-negative feedback loop.

2.4.4 DNA methylation studies in endometriosis

DNA methylation is one of the most studied epigenetic modifications of DNA, involved in regulation of many fundamentally important processes such as embryo development, gene expression, genome imprinting, X-chromosome inactivation, aging and disease development (reviewed Bird, 2002; Nasu et al., 2011). DNA methylation, where the cytosine nucleotide is converted by DNA methyltransferases into 5´-methylcytosine, occurs mainly in CpG dinucleotide rich areas (referred to as CpG islands). While the non-methylated CpG islands within the promoter region of the gene are required for the initiation of gene transcription, methylation of these CpG islands may result in down-regulation or silencing of the gene expression. The genome-wide studies have revealed that different cell types and tissues have their specific DNA methylation signature (Zhang et al., 2013) and alterations in this well-balanced system are suggested to be one possible molecular feature that contributes to the development of many human diseases, including endometriosis.

Recently, extensive progress has been made in identifying global changes in the DNA methylation profiles of eutopic endometria (Naqvi et al., 2014), eutopic/ectopic endometria (Borghese et al., 2010) and primary cell cultures of

eutopic and/or ectopic endometria (Dyson et al., 2014; Yamagata et al., 2014) from patients and controls (Table 3). Naqvi et al. investigated eutopic endometria of patients and controls and found 120 differentially methylated (1.5-fold or greater difference) genes (Naqvi et al., 2014). In the validation study, where authors included markers that have not been previously associated with endometriosis, hypermethylation of *MGMT*, *DUSP22*, *CDCA2*, *ID2* and hypomethylation of *BMPR1B*, *TNFRSF1B*, *ZNF681*, *IGSF21*, and *TP73* was confirmed. Although altered methylation levels of several previously known genes (*MAFB*, *HOXD10*, and *HOXD11*) were also confirmed, the different methylation in *PR-B*, *CYP19A1*, *SF1*, *COX2*, and *ER-β* genes that have been previously associated with endometriosis was not found.

DNA methylation profiles of endometriotic lesions or stromal cells originating from lesions have been described in three studies (Borghese et al., 2010; Dyson et al., 2014; Yamagata et al., 2014). The first genome wide DNA methylation study conducted by Borghese et al. (2010) utilized methylated DNA immunoprecipitation (MeDIP) arrays to profile specific promoters, and analysed DNA pools from ovarian endometriomas, DIE and SUP together with eutopic endometria (Borghese et al., 2010). They found a large number of differentially methylated regions in lesions (229 regions for SUP, 161 regions for endometriomas and 108 regions for DIE) compared to eutopic endometria. They found that overall methylation pattern in all three subtypes and eutopic endometria was similar; however, each subtype of the disease had specific regions that were consistently hyper- or hypomethylated. Combination of DNA methylation data with endometrioma gene expression data revealed no systematic correlation between these datasets; however, 35 genes with altered DNA methylation and expression were found and most of these were previously not associated with endometriosis.

A later study by Dyson et al. (2014), compared stromal cells obtained from ovarian endometriomas to stromal cells from eutopic endometria, and found more than four thousand differentially methylated CpGs (Dyson et al., 2014). When differentially methylated genes were compared to gene expression data, 403 genes with aberrant methylation and expression were identified, among them genes from the *HOXA* cluster, *ESR1*, *NR5A1* and *GATA* family transcription factors (Dyson et al., 2014). Interestingly, some of these genes such as *PRKAG2*, *HOXD10*, *ZNF22* and *ANO1*, were also reported in a former study by Borghese et al. (Borghese et al., 2010). Most recent study by Yamagata et al. (Yamagata et al., 2014) compared DNA methylation profiles between stromal cell cultures from endometriomas and eutopic endometria of patients and controls. They found 515 CpGs (441 genes) hypomethylated and 368 CpGs (329 genes) hypermethylated in stromal cells from endometrioma. The analysis of these genes revealed significant enrichment for multiple gene ontology (GO) terms and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways, such as biological processes of signal transduction, molecular functions of receptors

Table 3. DNA methylation studies in endometriosis

Microarray platform	Menstrual cycle phase	Studied tissues (n)	Results	Reference
MeDIP arrays	S	Pooled samples of 5 OMA, 5 SUP and 5 DIE samples,15 E	229, 161, and 108 differentially methylated regions in SUP, OMA, and DIE	(Borghese et al., 2010)
Illumina HumanMethylation450 BeadChip	P	SC from 6 OMA SC from 6 NE	5,423 differentially methylated CpGs	(Dyson et al., 2014)
Illumina HumanMethylation27 BeadChip	ND	SC from 3 OMA SC from 3 E, 3 NE	515 CpGs hypomethylated and 368 CpGs hypomethylated in OMA	(Yamagata et al., 2014)
Illumina HumanMethylation27 BeadChip	ND	7 E 6 NE	120 differentially methylated genes	(Naqvi et al., 2014)

P – proliferative, S – secretory, SC – primary cultures of stromal cells, E – endometriosis endometrium; NE – healthy or non-endometriosis endometrium, ND – not determined, OMA – endometrioma, SUP – superficial endometriotic lesion, DIE – deep infiltrating endometriotic lesions.

and signalling molecules, development and cytokine-cytokine receptor interactions. Although large numbers of differentially methylated genes were found between lesions and endometria, only few differences were seen between cells of eutopic endometrium of patients and controls. Based on the results, authors postulated that stromal cells from endometrioma are already differentiated into other cell types and have a specific fingerprint leading to abnormal cellular processes and development of the disease.

As all previous DNA methylation profiling studies varied in terms of study design, microarray platforms used, analysed samples and menstrual cycle phases of patients (Table 3) the concordance between the results of these studies has remained small and further studies, providing new evidence about the involvement of epigenetic alterations in disease pathogenesis, are needed.

2.5. Morphological and molecular changes in endometrium during the menstrual cycle

The endometrium is a unique layer of tissue inside the uterus that undergoes cyclic molecular, cellular and functional changes. The growth of endometrial tissue is under the firm control of endogenous hormones, cytokines and chemokines and during the normal menstrual cycle, which usually lasts 24–35 days, the fluctuation in sex hormone levels leads to cyclic growth and degeneration of endometrial tissue. The menstrual (uterine) cycle can be divided into three phases: menstrual, proliferative and secretory phase (Figure 9). The beginning of the menstrual bleeding is the first day of the menstrual cycle where the functional layer is shed and re-epithelisation in parallel with tissue breakdown is initiated (Ludwig et al., 1991). Proliferative phase is characterised by the rapid growth of endometrial glands, stroma and blood vessels and can be histologically separated into sub-stages: early, mid- and late proliferative (P) phase; however, this division is rarely used in endometrial biopsy dating as morphological changes in endometrial histology are very small (Mazur 2005). After ovulation (generally occurring on day 14 of a 28-day menstrual cycle), the growth of endometrial glands and stroma continues and reaches its maximum thickness during the mid-secretory (MS) phase (days 21–22), where the endometrial glands become tortuous and secretory active. During the late secretory (LS) phase (days 23–28), stromal cells increase in size and volume, acquire an epithelioid appearance and become predecidual cells. After that, endometrial regression begins and highly convoluted glands show apoptosis on day 27, which is followed by fibrin-thrombin formation in small vessels and haemorrhage with extravasation of erythrocytes into the stroma on day 28. At the beginning of the menstruation, the predecidual cells collapse and lose their abundant cytoplasm (Mazur 2005).

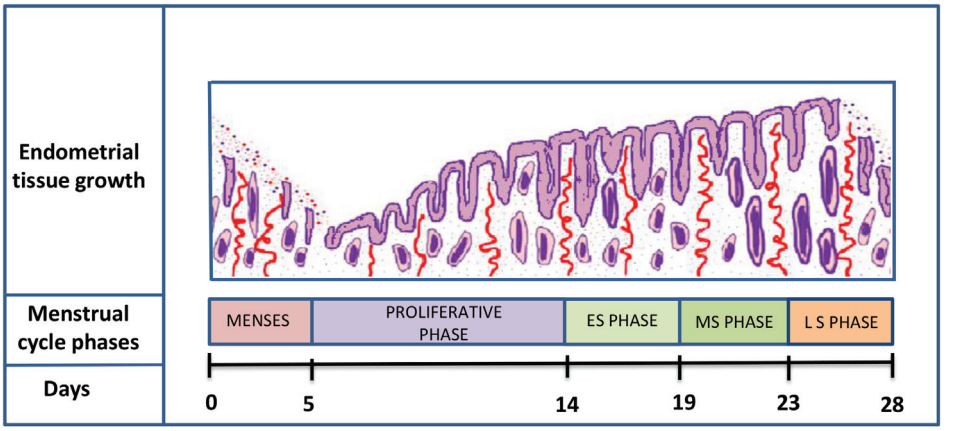

Figure 9. Changes in endometrial tissue structure during menstrual cycle. Menstrual cycle phases: ES – early secretory phase; MS – mid-secretory phase and LS – late-secretory phase.

As during the menstrual cycle significant changes occur in tissue morphology, it is evident that there should also be changes at the molecular level. Molecular profiling studies in endometria have focused mostly on secretory phase endometrium and tried to find transcriptome markers characteristic for window of implantation (Carson et al., 2002; Haouzi et al., 2009; Diaz-Gimeno et al., 2011; Sha et al., 2011; Hu et al., 2014). There are only a few studies both on transcriptome (Ponnampalam et al., 2004; Talbi et al., 2006) and epigenome level (Houshdaran et al., 2014) describing the molecular changes in healthy endometria throughout the menstrual cycle. Ponnampalam et al. and Talbi et al. studied the transcriptome of endometrial samples from different phases of menstrual cycle and found more than thousand differentially expressed genes across the menstrual cycle and reported a strong relationship between histological dating and molecular profile across the menstrual cycle. Furthermore, the results by Ponnampalam et al. emphasized that growth of endometrial tissue during the menstrual cycle is a continuum and grouping endometrial samples just based on the numerical menstrual cycle day will inevitably separate some very closely related samples into adjacent rather than similar groups (Ponnampalam et al., 2004). The involvement of epigenetic mechanisms in healthy human cycling endometrial tissue has been studied by Houshdaran et al. (2014), who demonstrated that the largest changes in DNA methylation occur between proliferative and mid-secretory phase where the endometrial tissue reaches its maximal thickness and is ready for embryo implantation (Houshdaran et al., 2014). They reported 66 differentially methylated CpG sites between MS and P, 27 CpG sites between ES and P and 22 CpG sites between MS and ES phases.

As the results from molecular profiling studies of healthy endometrium have provided clear evidence that endometrial transcriptome and epigenome are changing during the menstrual cycle, it is hard to under-estimate the importance of considering menstrual cycle phase also in studies investigating pathologic

conditions related to endometrial tissue. The significant impact of menstrual cycle phase on the transcriptome of endometrial tissue has also been reported in endometriosis studies (Burney et al., 2007; Aghajanova et al., 2011; Fassbender et al., 2012) and it has been proposed that the influence of menstrual cycle phase could have an even larger effect on endometrial transcriptome than the presence or absence of endometriosis (Aghajanova et al., 2011). Therefore, studies in eutopic- and ectopic endometria in endometriosis should carefully consider normal endometrial molecular changes that occur throughout the menstrual cycle to find disease-related changes.

3. AIMS OF THE STUDY

The general aim of the study was to find genomic markers that contribute to endometriosis development.

Accordingly, the specific aims were:

1. To explore, whether somatic *de novo* copy number alterations are present in endometriotic lesions and contribute to disease development.

2. To describe the miRNome of endometriotic lesions and to find miRNAs related to disease pathogenesis.

3. To reveal whether alterations in endometrial DNA methylation signature are common in endometriosis and could be the cause of disease development.

4. MATERIALS AND METHODS

The Research Ethics Committee of University of Tartu approved the study protocols and the informed consent forms. All participants signed a written informed consent.

4.1. Study participants

Patients undergoing laparoscopy for suspected endometriosis at the Women's Clinic of Tartu University Hospital (Tartu, Estonia, Study I, II and III), Elite Clinic (Tartu, Estonia, Study I, II and III) and John Radcliffe Hospital (Oxford, UK, Study III) were recruited into the study. All enrolled patients were in reproductive age and had received no hormonal medications at least three months before recruitment. The presence of endometriosis was confirmed during the laparoscopy by visual inspection and following histological examination of endometriotic lesions. The severity of endometriosis was classified according to the American Society for Reproductive Medicine revised classification system (ASRM, 1997). The general characteristics of all study subjects are presented in Table 4.

Table 4. General characteristics of study participants

	Study I	**Study II**	**Study III**
Discovery phase			
Patients with endometriosis (n)	11	2	31
Stage I–II (n)	3	–	19
Stage III–IV (n)	8	2	12
Studied tissues (type, n)	endometria, 11 lesions, 17 blood, 11	endometria, 2 lesions, 5 healthy tissue, 4	endometria, 31
BMI	23.4±3.3	22.5±0.5	22.3±2.9
Menstrual cycle phases	P (n=8), MS (n=1), ND (n=2)	P (n=1), LS (n=1)	M (n=4), P (n=2), ES (n=7), MS (n=9), LS (n=9)
Age	36.8±5.2	27.5±0.5	32.0±5.0

Table 4. Continuation

	Study I	Study II	Study III
Controls (n)	–	–	24
Studies tissues (n, tissue type)	–	–	endometria, 24
Menstrual cycle phases	–	–	M (n=1), P (n=3), ES (n=1), MS (n=17) LS (n=2)
BMI	–	–	24±4.4
Age	–	–	34.2±4.7
Validation study			
Patients with endometriosis (n)	187	30	15
Studied tissues from patients (type, n)	blood, 187	lesions, 22 non-diseased tissues, 24 endometria, 9	endometria, 15
Stage I–II (n)	94	11	13
Stage III–IV (n)	93	19	2
Menstrual cycle phase	NA	P (n=13), ES (n=3), MS (n=13), LS (n=5)	MS (n=7), LS (n=7)
BMI	NA	22.6±2.9	31.0±3.4
Age	32.6±6.1	32.4±5.0	20.0±3.9
Controls (n)	171	14	14
Studied tissues (type, n)	blood, 171	endometria, 8 stromal and epithelial cells from endometria, 5	endometria, 14
Menstrual cycle phase	NA	ES (n=3), MS (n=10)	MS (n=7), LS (n=8)
BMI	NA	21.7±2.6	32.0±2.7
Age	36.0±6.0	30.1±3.8	23.1±5.73

BMI is presented as mean, kg/m^2± standard deviation (SD) and age as mean years ± SD; NA, not applicable; M – menstrual phase; P – proliferative phase; ES – early secretory phase; MS – mid-secretory phase and LS – late-secretory phase.

Healthy women (n=171) representing the Estonian general population, with no medical history of endometriosis were enrolled as controls in Study I. Their genomic DNA isolated from blood samples was obtained from the Estonian Genome Center of the University of Tartu (Tartu, Estonia). Healthy controls for Study II (n=13) were recruited from Nova Vita Clinic (Tallinn, Estonia) and Elite Clinic. Healthy controls for Study III were recruited from Nova Vita Clinic (n=17), and from John Radcliffe Hospital (n=7). The healthy volunteers from Estonia were all in reproductive age (mean age 32, range 23–36 years), had at least one child from spontaneous pregnancy no more than 10 years ago, had not used hormonal medications three months before recruitment, had a regular menstrual cycle (28±5 days); normal serum levels of progesterone, prolactin, testosterone, had normal vaginal ultrasound, negative screening results for sexually transmitted diseases, no presence of endometriosis or polycystic ovary syndrome and were non-smokers. Controls from Oxford were endometriosis-free women undergoing laparoscopy for pelvic pain, subfertility or tubal sterilisation. They were in reproductive age (mean age 34, range 26–46 years) and had not used hormonal medication during previous three months.

4.2. Collection and processing of tissue samples

Peripheral EDTA-blood samples from patients were taken before surgery (Study I) and endometrial biopsy samples (Study I, II, III) from patients with endometriosis and healthy controls were obtained using an endometrial suction catheter (Pipelle, Laboratoire CCD, France). Endometriotic lesions and macroscopically healthy surrounding tissues adjacent to the lesions were removed during the laparoscopy. Tissue samples were either snap-frozen in liquid nitrogen and stored at −80 °C until use (Study I) or were immediately placed into RNA*later* (Ambion, Inc., Austin, Texas, USA) (Study II, III). After 24-hour incubation in RNA*later* at 4 °C, tissues were stored at −80 °C until use. DNA from blood and tissue samples was isolated using QIAamp DNA Mini kit (Study I) and Qiagen AllPrep DNA/RNA/miRNA Universal Kit (Study III). Total RNA together with miRNA-enriched fraction was extracted using MinElute Cleanup kit in combination with miRNeasy Mini kit (Study II).

4.3. Description of the study design

A graphical description of study design together with main findings is presented in Figure 10. Briefly, CNV profiling (Study I) was carried out at the Core Facility of the Estonian Genome Center, University of Tartu, using the HumanOmniExpress BeadChip (Illumina Inc., San Diego, CA, USA). DNA from matched samples of endometria, LCM-treated endometriotic lesions and peripheral blood originating from 11 endometriosis patients was examined.

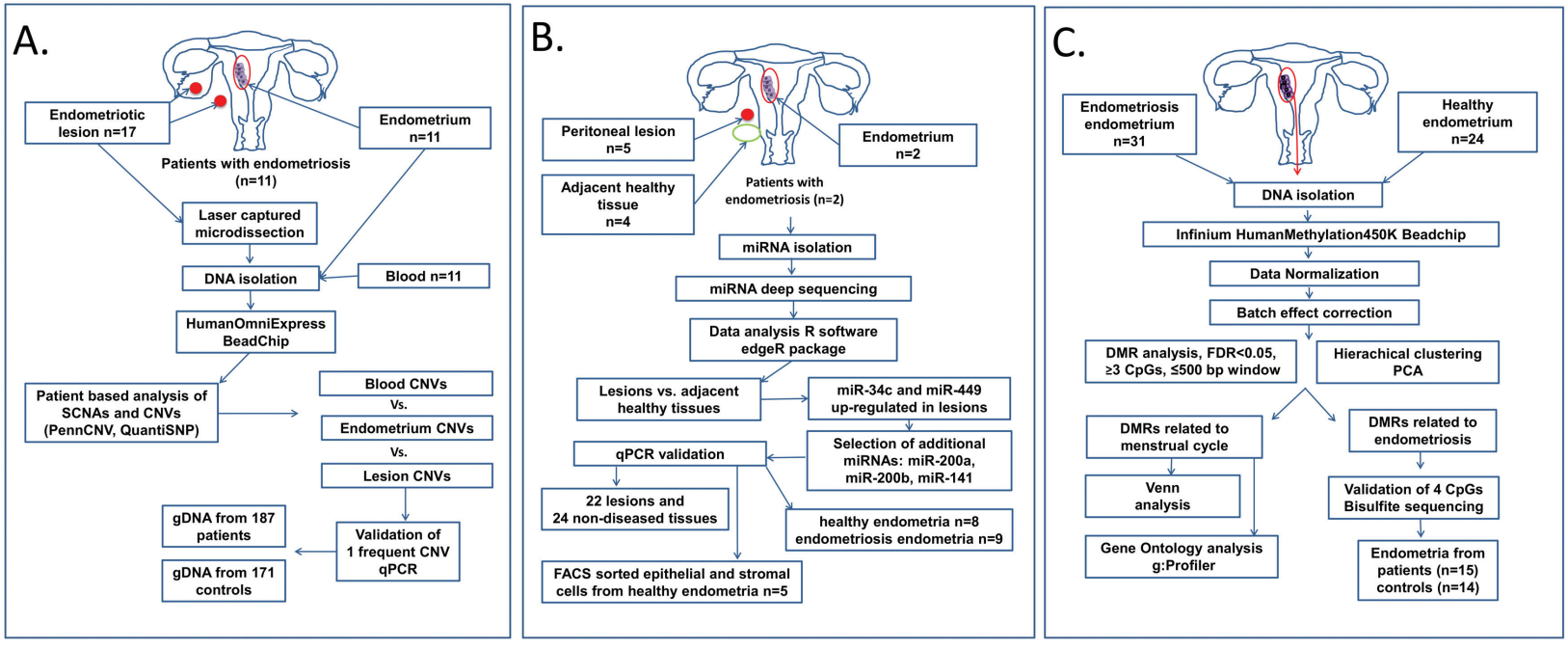

Figure 10. Graphical presentation of the study design. **A.** Study I, **B.** Study II and **C.** Study III.

The microarray data was analysed using GenomeStudio software, GT module v.3.1 (Illumina Inc), PennCNV (Wang et al., 2007) and QuantiSNP (Colella et al., 2007) programs. The presence of one CNV was validated in patients (n=187) and controls (n=171) by quantitative real-time PCR (qRT-PCR).

The miRNA profiling (Study II) from endometrial (n=2) and paired samples of peritoneal endometriotic lesions (n=5) and matched healthy surrounding tissues (n=4) was performed at Biomedicum Functional Genomics Unit (Helsinki, Finland) using Genome Analyzer IIx (Illumina). The sequencing data was analysed by miRDeep2 and FastQC (http://www.bioinformatics.babraham.ac.uk/projects/fastqc/) programs. The results were validated in an additional set of diseased (n=22) and non-diseased (n=24) tissues, in endometria of patients (n=9) and healthy women (n=8) and FACS sorted epithelial and stromal cells from five healthy endometria by qRT-PCR.

The DNA methylation profiling (Study III) from eutopic endometria of 31 patients and 24 controls was performed at USC Epigenome Center (Los Angeles, CA) by using the Infinium HumanMethylation450 BeadChips. The DNA methylation data was analysed with R statistical computing environment using Bioconductor package Minfi (Aryee et al., 2014) and ChAMP (Morris et al., 2012). The results were validated using additional endometrial samples from patients with endometriosis (n=15) and healthy women (n=14) by direct bisulfite sequencing.

The data analysis was performed in cooperation with Priit Palta (University of Tartu, Estonia), Jaak Simm (Tallinn University of Technology, Estonia), Tauno Metsalu (University of Tartu, Estonia), Triin Laisk-Podar (University of Tartu, Estonia) and Vijayachitra Modhukur (University of Tartu, Estonia).

4.4. Laser capture microdissection and histologic assessment of endometriotic lesions

For LCM (Study I), histological sections (10 μm) of the snap-frozen endometriotic lesion biopsies embedded in OCT (Leica, Germany) were mounted on PEN membrane (P.A.L.M. Mikrolaser Technology, Germany) microscope slides and stained with hematoxylin-eosin (HE) to confirm the presence of endometriotic cells. LCM of endometriotic epithelial and stromal cells from endometriotic lesions was performed using a PALM laser (MicroBeam, P.A.L.M. Mikrolaser Technology) (Figure 11). In Study II, histological assessment of endometriotic lesions was performed as follows: RNA*later*-preserved preserved tissue sections were embedded in OCT and cut (10 μm), mounted on standard microscope slides, stained with HE and evaluated histologically. The sectioning, examination and tissue collection for miRNA extraction was performed as follows: after the trimming, five to ten tissue sections were stained, examined and in case of a negative finding, the following 10–15 slices were collected into a microtube. The sectioning, examination and collection of the tissue was carried on until a positive finding (presence of endometrial epithelial and/or

stromal cells) or until all the sample was sectioned through (histologically negative finding). In case of a positive finding, the sectioning was stopped and the remaining biopsy was added to the tube containing previously collected tissue sections.

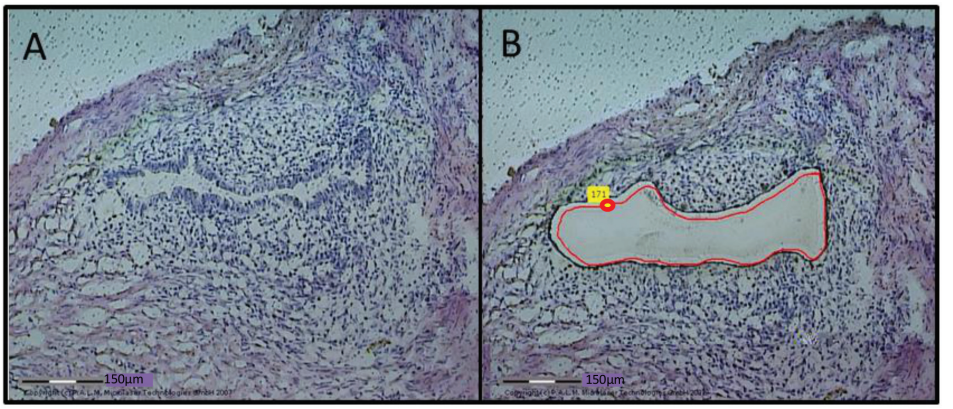

Figure 11. Representative photomicrographs of ectopic endometriotic lesions (section of *Ligamentum sacrouterina*, 10 × magnification, 10 µm HE stained section) before **A.** and after **B**. LCM.

4.5. Fluorescence-activated cell sorting (FACS)

To analyse the miRNA expression in a pure fraction of endometrial epithelial and stromal cells (Study II), the FACS sorted cell populations were obtained from five healthy women. Endometrial stromal cells were stained with fluorescence-conjugated rat anti-human CD13 monoclonal antibody (1:5 dilution, clone 1R3-63, R-Phycoerythrin, Novus Biologicals, Cambridge, UK) and epithelial cells with fluorescence-conjugated mouse anti-human CD9 monoclonal antibody (1:20 dilution, clone MEM-61, FITC, Novus Biologicals, Cambridge, UK). Target cell populations were sorted directly to QIAzol Lysis Reagent (Qiagen, Hilden, Germany) and total RNA was isolated immediately.

4.6. Validation studies using quantitative real-time PCR (qRT-PCR)

To detect hemi- and homozygous deletions in 3p14.1 region (Study I) the qRT-PCR reactions using 7500 Fast Real-Time PCR System (Applied Biosystems, Foster City, USA) were carried out using 20 ng DNA, 250 nM forward and reverse primers, 1 × HOT FIREPol EvaGreen qPCR Mix Plus (Solis BioDyne, Tartu, Estonia) and water in a total reaction volume of 20 µl per well. DNA copy number fold changes were calculated using the $2^{-\Delta\Delta Ct}$ method (Livak et al., 2001).

In Study II, five miRNAs: hsa-miR-449a (Applied Biosystems, Assay ID 001030), hsa-miR-34c-5p (ID 000428), hsa-miR-200a-3p (ID 000502), hsa-miR-200b-3p (ID 002251) and hsa-miR-141-3p (ID 000463) were selected for validation analysis (denoted hereafter as miR-449a, miR-34c, miR-200a, miR-200b, and miR-141). Two small nucleolar RNAs, RNU44 (ID 001094*)* and RNU48 (ID 001006) showing stable expression in human endometrium (Torres et al., 2013) were used as endogenous controls. cDNA synthesis was conducted from miRNA enriched RNA fraction with TaqMan® MicroRNA Reverse Transcription Kit (Applied Biosystems).

E-cadherin, *ZEB1* and *ZEB2* expression levels were determined in 12 paired endometriotic lesions' and adjacent healthy tissue samples. DNase treated (TURBO DNA-free™ kit, Ambion Inc., Austin, Texas, USA) RNA (up to 1 μg) was converted into cDNA using RevertAid First Strand cDNA Synthesis Kit (Thermo-Fisher Scientific Inc. MA, USA). qRT-PCR was performed using 1 × HOT FIREPol EvaGreen qPCR Mix Plus (Solis BioDyne) or TaqMan® Universal PCR Master Mix, No AmpErase® UNG (Applied Biosystems) and following the manufacturer's instructions.

4.7. Validation of DNA methylation array data by direct bisulfite sequencing

In Study III, four CpGs, two from *CST11* gene (cg06197930, cg12480562), one from *PI3* gene (cg19931348) and one from *SLC43A3* gene (cg13046608) were selected for validation analysis. Bisulfite modification of the endometrial DNA samples (500 ng each) was carried out with the EZ DNA Methylation-Gold™ kit (Zymo Research) according to the manufacturer's specifications. PCR primers for the bisulfite-treated DNA were designed using MethPrimer (Li et al., 2002). The sequencing results were analyzed as described in (Parrish et al., 2012) and using Mutation Surveyor software (Softgenetics, State College, PA, USA).

4.8. Statistical analysis

The Venn diagrams describing overlap between miRNA and transcriptome studies in endometriosis were created using the online tool InteractiVenn (Heberle et al., 2015) (http://www.interactivenn.net/).

The frequency distribution of the 3p14.1 deletion CNV from Study I was analysed using χ^2 test (PASW 18.0 software, SPSS, INC. USA).

In Study II, differential miRNA expression between endometriotic lesions, healthy tissues surrounding the lesions and eutopic endometria, was analysed using the program R (version 2.15.2) package edgeR (Robinson et al., 2010) available in Bioconductor version 2.8 was used. The p-values were adjusted for multiple testing using the Benjamini and Hochberg method. miRNA levels with false discovery rate (FDR)- adjusted p-values < 0.1 were considered statistically

significant. The multidimensional scaling (MDS) plots were created using edgeR. The Receiver Operating Characteristic (ROC) curve analysis was performed using MedCalc software version 12.7.5 (www.medcalc.org). For Clinical Score Formula (CSF) that classifies samples into healthy and endometriosis group based on the expression levels of miR-449a, miR-200a and miR-200b, linear soft margin Support Vector Machines classifier was trained using package e1071 in statistics language and environment R (version 3.0.1). The associations between the expression levels of *ZEB1*, *ZEB2*, E-cadherin and miR-200a, miR-200b and miR-141 were analysed by Pearson's correlation.

DMRs in Study III were identified using "seqlm" package https://github.com/raivokolde/seqlm) in the R environment, utilizing MDL based approach described earlier (Lokk et al., 2014). The Benjamini–Hochberg FDR was calculated for each probe, with a FDR corrected p-value <0.05 used to define DMRs. In order to get optimal DMRs, we limited our search in regions where distance between at least three consecutive probes was ≤ 500 bp. General characteristics and differences between methylation levels for patients and controls obtained from Sanger sequencing were calculated using Student's t-test, using a p-value cut-off of $p < 0.05$.

5. RESULTS

5.1. *De novo* somatic DNA copy number alterations in eutopic and ectopic endometrial tissue (Study I)

The CNV profiles obtained from LCM-treated endometriotic lesions and eutopic endometria were compared to those of blood from the same women in order to reveal *de novo* occurred SCNAs (study design is presented in Figure 10A). The analysis revealed no SCNAs while the endometriotic lesions from different locations (peritoneal lesions, superficial lesions from corpus uteri, ovarian superficial lesions and endometrioma) and endometria were analysed. All inherited CNVs that were present in blood and endometrium were also detected in endometriotic lesions, confirming the reliability of LCM DNA for SCNA detections by SNP array.

5.2. Genomic DNA copy number alterations in endometriosis (Study I)

In order to find genomic inherited CNVs (present in all tissues of the same individual) that could be more frequently present in endometriosis patients, CNVs of all 11 patients were analysed. Altogether 52 (0–11 per person) genomic CNVs with an average region size of 85 kb (ranging from 3 kb to 340 kb) were found in matched blood, endometrial and ectopic lesions samples. Most of CNVs (n=48) were described in the DGV and therefore considered as common CNVs. Four CNVs were novel: two hemizygous microdeletions (2p21 and 14q12) and two hemizygous duplications (1q42.3 and 10q23.1). Five CNVs (2p22.3, 3p14.1, 4q13.1, 5q15, 10q11.22) were present in more than one patient. As the frequency of the deletion in 3p14.1 was considerably higher in our patients than reported in the DGV (45% vs. 8%), the presence of this CNV was validated in blood DNA of additional 176 endometriosis patients, and 171 healthy women from the Estonian general population. However, there were no significant differences between the frequency of deleted allele in the patient and control group (9.4% vs. 6.4%, $p \geq 0.05$).

5.3. miRNA profile of endometriotic lesions, surrounding healthy tissues and endometrial tissues (Study II)

In the discovery phase, miRNAs of five peritoneal endometriotic lesions, four matched adjacent healthy tissues (two lesions originating from the same ligament were matched with the same adjacent healthy tissue) and two eutopic endometrial samples from two women with severe endometriosis were sequenced using Illumina high throughput sequencing technology (study design presented

in Figure 10B). The analysis of miRNA sequencing data showed remarkable overlap between miRNA expression patterns of all lesions and adjacent healthy tissues of the same patient. The MDS plot (Figure 12) demonstrated that all studied tissue pairs of the same patient were closer to each other than to the respective endometrium originating from the same patient. Based on the MDS plot data it was decided to exclude eutopic endometria from future analysis and for determination of lesion-specific miRNA profile, to use only endometriotic lesions and healthy adjacent tissues. To reveal miRNAs differently regulated in lesions, all lesions were compared to all healthy tissues. The comparison identified two miRNAs: miR-34c (FDR adjusted p-value 0.005, FC =16.7) and miR-449a (FDR adjusted p-value 0.005, FC = 20.1) that were significantly up-regulated in endometriotic lesions compared to healthy tissues. The counts of both miRNAs in lesions and healthy tissues were remarkably lower than in eutopic endometria.

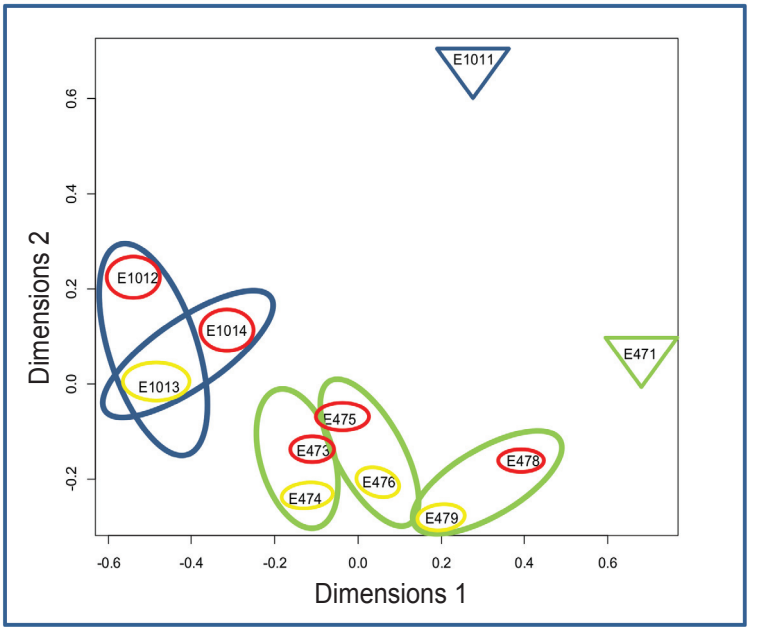

Figure 12. Clustering analysis of the miRNA counts in eutopic and ectopic tissue samples. Dimension 1 represents different tissue types (endometria, healthy tissues, and endometriotic lesions) while dimension 2 denotes different patients. Blue and green colours indicate different patients. Red circles represent endometriotic lesions, yellow circles adjacent healthy tissues and triangles with patient-specific colour denote respective endometria. The tissue samples presented in figure are following: patient E101 (blue) – endometrium E1011, lesions E1012, E1014, matched healthy tissue E1013; patient E47 (green) – endometrium E471, lesions E473, E475, E478, matched healthy tissues E474, E476, E479.

5.4. Validation of altered expression of miR-34c, miR-449a and miR-200 family in endometriotic lesions, endometrium and FACS sorted epithelial and stromal cells (Study II)

In addition to miR-34c and miR-449a, the sequencing data of patient E47 samples showed trend to higher expression of miR-200a, miR-200b and miR-141 in lesions compared to healthy tissues. As previous publications (Filigheddu et al., 2010; Hawkins et al., 2011; Braza-Boils et al., 2014; Shi et al., 2014) have also proposed altered expression of miR-200 family members in endometriosis, these three miRNAs were additionally selected for validation analysis. The expression differences of miR-449a, miR-34c and members of miR-200 family between peritoneal endometriotic lesions and healthy tissues were determined in 22 diseased and 24 non-diseased tissue samples (Figure 13). Validation analysis confirmed the results obtained from miRNA sequencing and all studied miRNAs showed significantly higher expression in endometriotic lesions (FC for miR-449a = 95.7, miR-34c = 5.9, miR-200a = 6.0, miR-200b = 5.7 and miR-141 = 11.2) compared to healthy tissue samples (all $p < 0.0001$). The results of qRT-PCR indicated that adjacent healthy tissues and histologically unconfirmed lesion-like tissue samples had very similar expression levels of the tested miRNAs (all $p > 0.05$) and therefore it was decided to combine both types of samples as "non-diseased" group. When expression data was evaluated in the combined group, even more significant differences were seen between lesions and the non-diseased tissue group (FC for miR-449a = 129.9, miR-34c = 10.2,

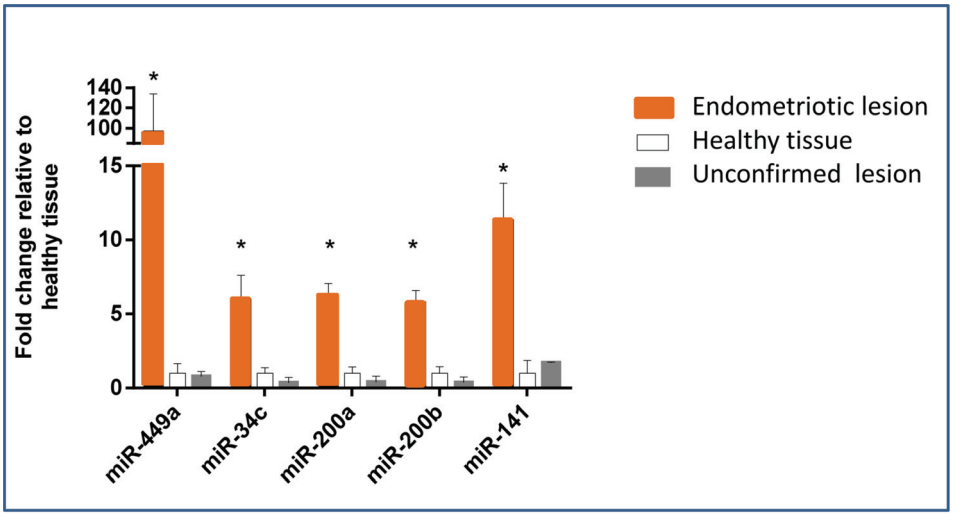

Figure 13. The expression of selected miRNAs in endometriotic lesions (n=22), histologically unconfirmed lesions (n=10) and healthy tissues (n=14) using qRT-PCR. The error bars denote SEM (standard error of the mean). * Denotes comparison of endometriotic lesions with healthy tissues, $p < 0.0001$.

miR-200a = 10.5, miR-200b = 10.2 and miR-141 = 16.8; $p < 0.0001$). To test whether the expression of these miRNAs is differently regulated in the endometrium of endometriosis patients, the endometria of nine patients with endometriosis and eight healthy women were analysed. However, the results revealed no significant expression level differences between the endometria of endometriosis patients and controls (all $p > 0.05$).

Furthermore, to confirm epithelial cell-specific expression pattern of these miRNAs, FACS-separated endometrial epithelial and stromal cells originating from five endometria of healthy women were used (Figure 14). Data analysis revealed significantly higher expression of studied miRNAs in epithelial cells compared to stromal cells (FC for miR-449a = 86.1, miR-34c = 16.7, miR-200a = 52.4, miR-200b = 44.6 and miR-141 = 68.4; $p < 0.0001$) indicating an epithelial cell-specific miRNA expression pattern.

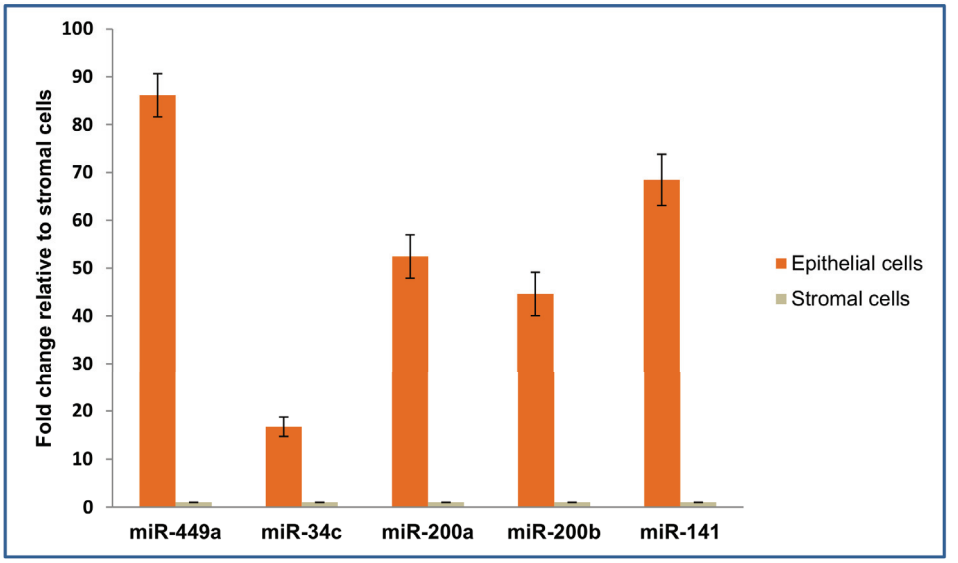

Figure 14. The expression of miRNAs in FACS sorted epithelial and stromal cells using qRT-PCR. The error bars denote SEM.

5.5. E-cadherin, *ZEB1* and *ZEB2* expression levels in endometriotic lesions and adjacent healthy tissues (Study II)

As the known target genes of the miR-200 family are E-cadherin, *ZEB1* and *ZEB2*, mRNA expression levels of these genes were studied between endometriotic lesions and adjacent healthy tissues in 12 paired tissue samples. E-cadherin showed statistically higher expression in endometriotic lesions (t-test, $p = 0.01$) but there were no significant differences in *ZEB1* and *ZEB2*

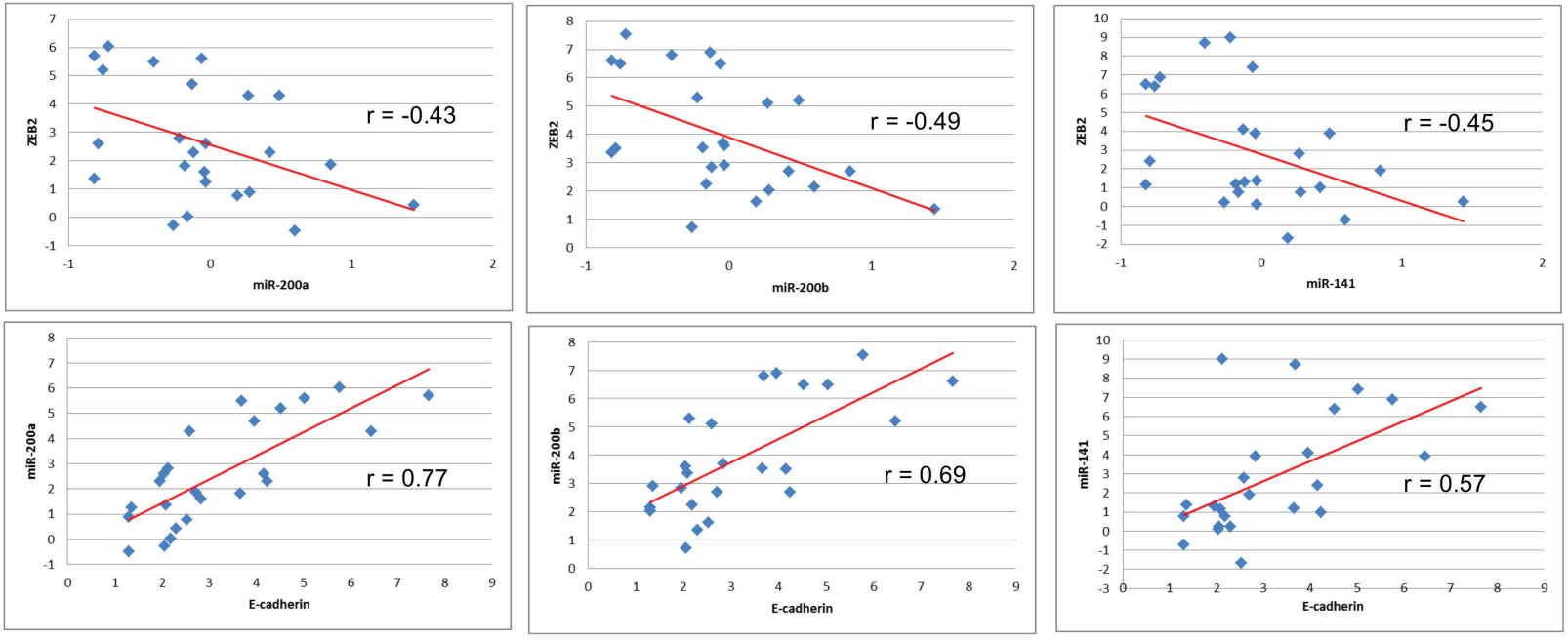

Figure 15. Correlations between expression levels of miR-200a, miR-200b and miR-141 and *ZEB2* and E-cadherin, with the p-values provided in the text. r – correlation coefficient.

expression levels between the diseased and non-diseased samples (t-test, $p > 0.05$). The correlation analysis (Figure 15) showed a significant negative correlation between *ZEB2* and miR-200a ($r = –0.43$, $p = 0.03$), miR-200b ($r = –0.49$, $p = 0.01$), and miR-141 ($r = –0.45$, $p = 0.02$) expression, and a positive correlation between E-cadherin and miR-200a ($r = 0.77$, $p < 0.001$), miR-200b ($r = 0.69$, $p < 0.001$), and miR-141 ($r = 0.57$, $p = 0.02$) expression. *ZEB1* mRNA levels did not correlate with miR-200 family expression levels.

5.6. Diagnostic potential of miR-34c, miR-449a, miR-200a, miR-200b and miR-141 (Study II)

As the level of these miRNAs was significantly higher in endometriotic lesions than in healthy tissues, the ability of these miRNAs to discriminate endometriotic lesions (n=22) from non-diseased tissues (n=24) was evaluated. The ROC analysis revealed that all miRNAs enabled a correct classification of diseased and non-diseased tissues (area under the curve – AUC values for miR-449a, miR-34c, miR-200a, miR-200b and miR-141 were 0.92, 0.81, 0.91, 0.90, and 0.83, respectively). The combined signature of five miRNAs showed high sensitivity (91.7%) and specificity (95.5%), (AUC = 0.94, CI 0.83–0.99) but the subset of three miRNAs – miR-449a/miR-200a/miR-200b – showed the best discriminating power with the highest sensitivity (95.8%) and specificity (95.5%) (AUC = 0.95, CI 0.83–0.99).

Based on the expression levels of miR-449a, miR-200a and miR-200b, the Clinical Score Formula (CSF) score (CSF score = $X \times \Delta Ct$ of miR-449a + $Y \times \Delta Ct$ of miR-200a – $Z \times \Delta Ct$ of miR- 200b – W) was calculated. In CSF score X, Y, Z and W denote the coefficients determined by the classifier algorithm which are based on the already analysed tissue samples, while ΔCt is a threshold value difference of target and reference miRNAs of the study samples. More specifically, X, Y and Z (parameters for each miRNA) and W (intercept) are found by training a Support Vector Machine classifier which finds a linear combination of the miRNAs that best separates endometriotic lesions from healthy tissues. Based on the CSF score, values lower than 0 indicate that the studied tissue samples are endometriotic lesions, and a score above 0 indicates that the studied tissue samples are disease-free. The CSF score enabled to distinguish endometriotic tissue samples from lesion-like samples with very high classification accuracy (> 95%).

5.7. DNA methylation in endometria of endometriosis patients and controls (Study III)

Endometrial samples from 31 endometriosis patients and 24 disease-free women, from M (n=5), P (n=5), ES (n=8), MS (n=26) and LS (n=11) menstrual cycle phases were used for genome-wide DNA methylation analysis. Study

design is presented in Figure 10C. The principal component analysis (PCA) clustering of the normalized data showed no significant segregation between patients and controls indicating that the overall DNA methylation profile between patients and controls was very similar (Figure 16).

Nevertheless, if we compared the methylation profiles of all patients with endometriosis to healthy women, we found 28 DMRs (FDR <0.05, Δβ ranging from -0.01 to -0.16 and from 0.01 to 0.08) from which 16 were associated with known genes (*PI3*, *SLC43A3*, *MGAT5B*, *MUC4*, *HIVEP3*, *FGG*, *CLCF1*, *CANT1, LTK*, *AHRR*, *AKR1B1*, *APEH*, *CST11*, *ELOVL4*, *HBE1* and *NEGR*1, Table 5). Twelve DMRs were not related to any genes and one of the top-ranking intergenic DMRs was located on chromosome locus 7p15.2, about 13 kb upstream from *HOXA* gene cluster. After adjusting DNA differential methylation analysis for different variables like age, nationality, menstrual cycle phase and BMI, no DMR remained statistically significant. Menstrual cycle phase had the most significant impact on DNA methylation profile according to multivariable analysis.

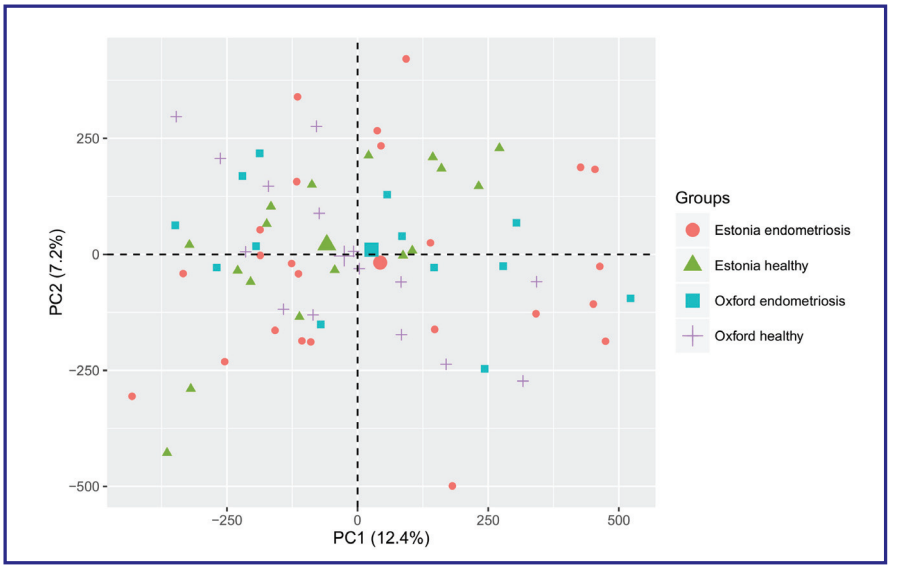

Figure 16. Principal component analysis describing DNA methylation data across all studied endometrial samples from Estonia and Oxford. The large dots and triangles mark overlapping samples.

To confirm the results of microarray analysis, four CpG sites located in the promoter regions (two CpGs from *CST11* gene, one from *PI3* gene and one from *SLC43A3* gene) were selected for validation analysis by conventional bisulphite Sanger sequencing in an extended group of patients and controls from LS (n=15) and MS phase (n=14). The correlation analysis between microarray and bisulphite sequencing data showed strong correlation (Pearson correlation coefficient, PCC > 0.85, $p < 0.001$) for *SLC43A3* and *CST11* and moderate

correlation (PCC = 0.58, p = 0.07) for *PI3*. From four analysed CpG sites, only the CpG from *SLC43A3* gene showed statistically significant differential methylation between MS patients and controls (p = 0.03).

Tabel 5. Gene- associated DMRs between women with and without endometriosis

Chromo-some	Start	End	CpGs in region	Gene name	FDR	Average Δβ
1	41981789	41982027	4	*HIVEP3*	0.008	–0.01
17	74928552	74928737	3	*MGAT5B*	0.020	0.03
11	57195025	57195382	4	*SLC43A3*	0.020	0.03
20	43802992	43803485	3	*PI3*	0.020	0.04
3	195488493	195488922	3	*MUC4*	0.020	–0.12
3	195489306	195489782	3	*MUC4*	0.040	–0.05
5	434877	435267	4	*AHRR*	0.020	0.20
17	76991208	76991378	3	*CANT1*	0.030	–0.01
3	49710768	49711095	4	*APEH*	0.030	0.06
1	72748607	72749064	4	*NEGR1*	0.030	–0.004
20	23434086	23434279	3	*CST11*	0.030	0.03
7	134144106	134144288	3	*AKR1B1*	0.030	0.02
4	155533784	155534149	3	*FGG*	0.030	0.04
15	41803428	41803850	4	*LTK*	0.030	0.03
11	5291214	5291692	3	*HBE1*	0.040	0.06
11	67141747	67141961	3	*CLCF1*	0.040	–0.003
6	80657412	80657436	5	*ELOVL4*	0.040	0.02

5.8. Menstrual cycle-specific DNA methylation signature of endometrium (Study III)

Interestingly, unsupervised hierarchical clustering analysis of methylation data revealed two main branches that divided endometrial samples based on the menstrual cycle phase rather than diseased/non-diseased status (Figure 17). The first branch included all LS phase samples (n=11), four out of five M phase (n=4) and some MS phase (n=7) samples, while the other branch included all other samples (19 from MS, 8 from ES, 5 from P and one remaining sample from M phase).

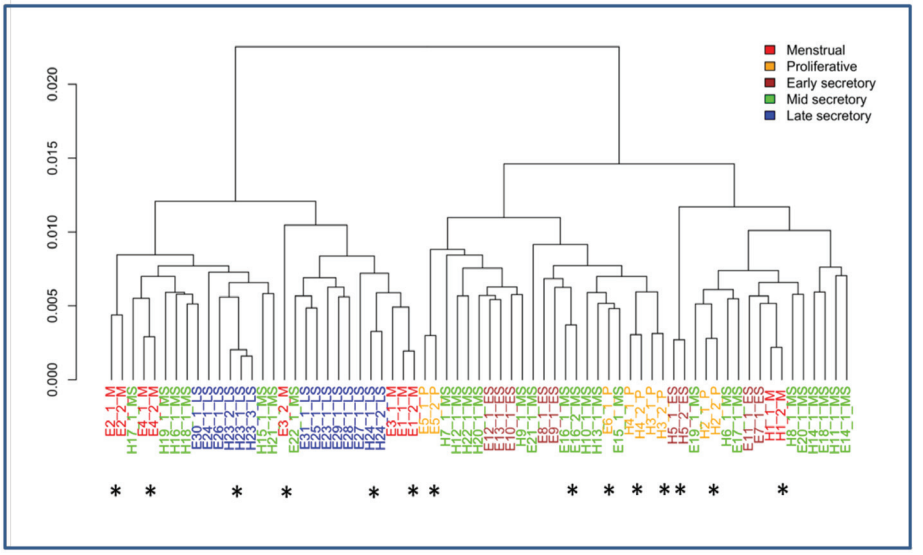

Figure 17. Hierarchical clustering analysis of all endometrial samples included into the study. Sample codes starting with E indicate patients with endometriosis and H indicates healthy individuals. Samples with the same index number are duplicates. The asterisk indicates samples from the Oxford cohort.

As there was no segregation between patients and controls, both groups were combined (altogether 55 individuals) to find menstrual cycle phase-specific methylation changes. The studied individuals were divided into five groups (M, P, ES, MS and LS phase) according to the menstrual cycle day at the time of biopsy collection and to assess the overall methylation pattern characteristic to each cycle phase the methylation data of each phase was compared to other phases. A large number of differentially hypo- and hypermethylated regions (FDR < 0.05) were noticed when either or both M and LS phases were involved in comparisons, while only some DMRs were found in comparisons between P, ES and MS phases (Table 6).

While menstrual cycle phase comparisons revealed several differences in the methylation pattern, we compared the lists of endometriosis-specific differentially methylated genes and regions to the menstrual cycle-specific alterations. Results showed that eight out of 16 differentially methylated genes found in patients with endometriosis overlapped with the menstrual cycle-related genes: seven genes (*PI3*, *SLC43A3*, *MGAT5B*, *MUC4*, *HIVEP3*, *FGG* and *CANT1*) from comparison between MS and LS phase and one gene (*LTK*) from M to P comparison. The remaining eight differentially methylated genes – *AHRR*, *AKR1B1*, *APEH*, *CST11*, *ELOVL4*, *CLCF1*, *HBE1* and *NEGR*1 were not related to the menstrual cycle changes. From DMRs that were not related to any genes, five were also found in the lists of menstrual cycle-specific loci.

Table 6. The number of differentially hyper-/hypomethylated DMRs and genes between menstrual cycle phases

	The number of DMRs and genes[a] (hyper-/hypomethylated)				
	M (n=5)	**P (n=5)**	**ES (n=8)**	**MS (n=26)**	**LS (n=11)**
M (n=5)		1009/1775 632/1066	130/189 92/116	363/855 254/512	288/368 208/222
P (n=5)	1009/1775 632/1066		0/0 0/0	1/2 1/0	3045/1650 1768/936
ES (n=8)	130/189 92/116	0/0 0/0		0/5 0/3	2806/1208 1015/635
MS (n=26)	363/855 254/512	1/2 1/0	0/5 0/3		2806/1208 1616/704
LS (n=11)	288/368 208/222	3045/1650 1768/936	1727/1050 1015/635	2806/1208 1616/704	

[a] The number of DMRs is given in the upper, and the number of differentially methylated genes in the lower row of the respective comparison of the menstrual cycle phases.

6. DISCUSSION

In our studies we have used genome-wide approaches to take a deeper look into the molecular architecture of endometriotic lesions and endometria of endometriosis patients. We discovered not only new molecular aspects about the disease but also brought out several important concerns related to proper study design in endometriosis research. In the following sections the relevance of the findings in the context of study design, disease pathogenesis and molecular diagnostics will be discussed.

6.1. Relevance of the findings in the context of study design in endometriosis research

It has been seventeen years since the first endometriosis large-scale molecular profiling study was conducted in 1999 (Gogusev et al., 1999). Since then, the fast development of new technologies has generated opportunities to get novel insights into the complexity of endometriosis at the molecular level. About fifty endometriosis studies have used modern, high-throughput technologies to uncover the potential mechanisms of disease development but regardless of the numerous studies, only slight progress has been made towards uncovering the mystery of endometriosis.

One of the possible reasons why these attempts have not yet answered the question why endometriosis develops may be in the low concordance between the results of similar studies. Discoveries made in one study are rarely reproduced by others, most probably because of the variable approaches towards study design. Inconsistencies in endometrial and endometriotic lesion biopsy collection, processing, and storage methodology, insufficient reports of patient phenotype data, small sizes of study groups and differences between microarrays, together with variations in data analysis and interpretation, are the major affecting factors that could lead to biases and measurement errors between studies. Recently, the World Endometriosis Research Foundation Endometriosis Phenome and Biobanking Harmonisation Project has made an enormous effort to harmonize the standard operating procedures for collection of patients' phenotype data, tissue samples, and processing and storage of biospecimens (Becker et al., 2014; Fassbender et al., 2014; Rahmioglu et al., 2014a), and the proposed guidelines would definitely improve the quality of endometriosis molecular profiling research.

The results of our studies have added three very important concerns related to study design in endometriosis research. Firstly, the results of Study I accentuated the relevance of considering the individual-specific blood CNV profile while examining somatic disease-related changes. Previously, in (a)CGH analyses the reference DNA with unknown absolute copy numbers of given CNVs has been used to find SCNAs in lesions and endometria. In our study, we

compared each patient's blood to eutopic and ectopic endometria to eliminate inter-individual variability in CNVs. Comparisons revealed a number of different inherited CNVs (ranging from 0 to 11) in each studied individual and this is in good agreement with previous reports suggesting a presence of a variable number of CNVs in normal human genome (Sebat et al., 2004; Pinto et al., 2007; Teo et al., 2011; Li et al., 2013). It is possible that some of the alterations reported in previous endometriosis studies actually represent CNVs that are present in all tissues of studied individuals, rather than endometriotic lesion-specific SCNAs. Based on our results, we suggest that instead of pooled reference DNAs, genomic DNA from blood should always be used to determine the background information of individual CNVs while searching SCNAs from endometriotic lesions.

Secondly, in Study II we saw a significant impact of studied tissue-content on miRNA signature. In studies investigating endometriotic lesions, little attention has been paid to cellular composition of lesions and majority of endometriosis studies have used entire biopsied samples where the proportion of surrounding tissue is variable and may even be larger than the amount of endometrial epithelial and stromal cells. Our data demonstrate that miRNA profiles of endometriotic lesions were more similar to those originating from the peritoneal tissue than those from respective endometria, meaning that comparison of two different tissues (endometrium vs. lesion that is a mixture of endometrial and peritoneal cells) with their own specific miRNA profiles brings out differences between tissue types rather than differences related to the disease. However, although the miRNA profile of peritoneal endometriotic lesions was largely masked by the surrounding peritoneal tissue, we were able to find a signature of five miRNAs that were more highly expressed in endometriotic lesions compared to macroscopically healthy peritoneal tissue. Further analysis confirmed that these five miRNAs (miR-200a, miR-200b, miR-141, miR-449a and miR-34c) were more highly expressed in endometrial epithelial cells and therefore it was concluded that higher expression levels of these miRNAs are not related to different characteristics of endometrial cells in eu- or ectopic locations, but rather reflect the presence of endometrial epithelial cells in peritoneal tissue. Therefore, based on our results and the fact that tissue-specific variability in miRNA levels (Vrba et al., 2011), DNA methylation (Zhang et al., 2013; Lokk et al., 2014) and in gene expression does exist, the normal variability between different tissue types should be taken into account while searching molecular markers for endometriosis. Tissue-specific changes may partially explain the discordances in results between molecular profiling studies where entire lesion tissue biopsies from distinct locations (peritoneal, ovarian, intestinal etc.) are compared to eutopic endometria, and this issue should be taken into account when interpreting the results of such studies.

Thirdly, in Study III, a significant influence of menstrual cycle phases on endometrial methylome was noticed. There is evidence from transcriptome (Ponnampalam et al., 2004; Talbi et al., 2006) and epigenome (Houshdaran et al., 2014) studies that molecular changes in healthy endometria occur throughout

the menstrual cycle. Also, menstrual cycle-specific changes have been found in endometriosis transcriptome studies (Burney et al., 2007; Aghajanova et al., 2011; Fassbender et al., 2012) but their influence on endometrial epigenome has not been considered in endometriosis research. As the tissue samples from previous epigenome studies in endometriosis were either from proliferative (Dyson et al., 2014) or secretory (Borghese et al., 2010) phase or with no information about the menstrual cycle (Naqvi et al., 2014; Yamagata et al., 2014), it could be that at least some of the reported differences between lesions and endometrium may arise from the variability of samples in respect of menstrual cycle phases. We found a large number of DMRs between samples from different menstrual cycle phases, accentuating the importance of considering normal cyclic epigenetic changes while searching disease-specific molecular markers. Based on our data and previous reports, we suggest that menstrual cycle phase is an extremely important issue that should be taken into account while planning molecular profiling studies and not only in eutopic endometria but also in endometriotic lesions.

Taken together, the results of our studies highlighted the relevance of proper study design that could have a significant impact on the overall outcome and interpretation of the results. We believe that the new knowledge about the normal variability in CNVs, tissue-specific molecular patterns and impact of menstrual cycle on the molecular profile of endometrial tissue, will help to clarify the global picture of the disease.

6.2. Relevance of the findings in the context of endometriosis pathogenesis

All studies presented in this thesis were aimed to search for potential mechanisms that are relevant in endometriosis pathogenesis. Study I was driven by the idea that *de novo* occurred SCNAs are associated with disease pathogenesis. In Study II we searched for the disease-specific miRNA signature from lesions and in Study III we investigated epigenetic alterations in endometria to shed light on the molecular events triggering the disease development. However, the results of Study I contradicted the majority of the preceding studies reporting the presence of unique SCNAs in endometriotic lesions (Gogusev et al., 1999; Wu et al., 2006b; Veiga-Castelli et al., 2010; Silveira et al., 2012; Li et al., 2014) as we found no SCNAs associated with endometriosis development. The most probable reason explaining our discordant results may again be hidden in the methodological differences. Most studies reporting large numbers of somatic alterations have used CGH or aCGH methods (Gogusev et al., 1999; Wu et al., 2006b; Veiga-Castelli et al., 2010; Silveira et al., 2012; Yang et al., 2013). Although CGH is a valuable method for discovering SCNAs, it has been shown that some G-C-rich chromosomal regions (1p, 16p and chromosomes 19 and 22) tend to give false-positive results (Karhu et al., 1997; Veiga-Castelli et

al., 2010). Also, it could be that some of the genomic alterations reported in earlier studies are caused by whole genome amplification (WGA) used for multiplying the minute amounts of LCM DNA prior to CGH (Wu et al., 2006b; Silveira et al., 2012). WGA-generated amplification artefacts can create false deletions and duplications and therefore it is not an ideal method for DNA amplification before CGH arrays (Talseth-Palmer et al., 2008). In our study the SNP genotyping array was used where the amount of required DNA is considerably smaller than in (a)CGH and therefore there was no need to use WGA. Furthermore, we cannot exclude the possibility that differences in findings might be caused by the selection of studied tissue samples as there is evidence from ovarian cancer studies that at least some of endometriotic lesions may be premalignant with diverse malignant potential (Oral et al., 2003). However, based on our results we believe that molecular mechanisms other than chromosomal rearrangements most likely underlie the initiation and progression of endometriosis.

In Study II we detected five miRNAs- miR-34c, miR-449a, miR-200a, miR-200b and miR-141 which were significantly up-regulated in endometriotic lesions compared to healthy tissues. Interestingly, almost all these miRNAs (except for miR-449a) have been repeatedly associated with endometriosis pathogenesis (Figure 7B). miR-34c and miR-449a, are classified as one family because of their highly similar secondary structures and seed sequence. It has been suggested that members of this family have tumor-inhibitory effects and are capable of mediating cell cycle arrest and apoptosis via the p53 signalling pathway (reviewed in Lize et al., 2011). While the relevance of miR-449a in endometriosis has not been described earlier, the altered expression levels of miR-34c in endometriotic lesions and also in eutopic endometrium of endometriosis patients have been mentioned (Burney et al., 2009; Ohlsson Teague et al., 2009; Hawkins et al., 2011) but the results of our study did not confirm differential expression of miR-34c between eutopic endometria of patients and controls. Therefore, the potential role of this miRNA in endometriosis pathogenesis is currently unclear.

Further, we found that miR-34c, miR-449a and members of miR-200 family were more highly expressed in FACS-sorted endometrial epithelial cells than in stromal cell. The higher expression of miR-200 in epithelial cells is in good concordance with previous reports showing that members of the miR-200 family are actively involved in maintaining the epithelial nature of cells (Park et al., 2008) and are involved in regulation of EMT and its reverse process, MET (reviewed in Hill et al., 2013). Previous studies have shown significant down-regulation of these miRNAs in endometriotic lesions compared to endometrial tissue (Ohlsson Teague et al., 2009; Filigheddu et al., 2010; Hawkins et al., 2011). The recent functional study using endometriotic cell lines has confirmed that up-regulation of miR-200b results with down-regulation of *ZEB1* and *ZEB2* and up-regulation of E-cadherin that in turn leads to decreased cell invasiveness and motility (Eggers et al., 2016). In our study, the gene expression of miR-200 targets *ZEB1/ZEB2* was not different in lesions compared to healthy tissues but

E-cadherin showed significantly higher expression. However, the correlation analysis showed negative correlation between miR-200 family members and *ZEB2* levels and positive correlation with E-cadherin, confirming the effect of miR-200 family to *ZEB1/ZEB2* expression. Nevertheless, as our results showed that miRNA expression levels of surrounding tissue mask those in ectopic endometrial cells, we suggest that pure populations of endometrial and endometriotic cells should be explored to determine, whether and how these miRNAs are associated with endometriosis pathogenesis.

Genome-wide DNA methylation studies have suggested that the abnormal DNA methylation pattern in endometria and lesions of endometriosis patients could be one potential cause leading to the development of endometriosis (Borghese et al., 2010; Dyson et al., 2014; Naqvi et al., 2014; Yamagata et al., 2014). The results from Study III demonstrated that the epigenetic signature of endometrial tissue in patients and controls was highly similar and endometrial samples clustered according to the menstrual cycle phase rather than diseased/non-diseased status. However, we found 28 DMRs between patients with endometriosis and controls when menstrual cycle phases were not taken into account. Sixteen of them were located in known genes and from those eight genes (*PI3*, *SLC43A3*, *MGAT5B*, *MUC4*, *HIVEP3*, *FGG*, *CANT1* and *LTK*) were also among the menstrual cycle phase-dependent genes. The remaining eight genes (*AHRR*, *AKR1B1*, *APEH*, *CLCF1*, *CST11*, *ELOVL4*, *HBE1* and *NEGR1*) could be attractive candidate genes for endometriosis; however, none of the previous DNA methylation studies have reported differential methylation of these genes in association with endometriosis. Additionally, it should be pointed out that the overall magnitude of the methylation differences was rather small. How small-scale changes in DNA methylation level are associated with the disease pathogenesis, what is their biological significance and whether they are the cause or the consequence of the disease, is currently unknown. Furthermore, it is not clear how the small-scale DNA methylation changes could affect gene expression. When we compared our results to a gene expression study by Tamaresis et al. (Tamaresis et al., 2014), it was evident that several genes showing differential methylation in our study (such as *AHRR*, *APEH*, *ELOV4*, *PI3*, S*LC43A3*, *MUC4*, *CANT* and *CLCF1*) revealed also differential expression between patients and controls from certain menstrual cycle phases.

When looking at differentially methylated genes in endometriosis, we saw some genes that might be interesting from a biological point of view, such as *AKR1B1*, *AHRR* and *SLC43A3*. *AKR1B1* catalyses the formation of prostaglandin F2α (PGF2α), and is shown to be widely expressed in normal endometrium and myometrium (reviewed in Rizner, 2012). Concurrently increased synthesis of prostaglandin PGF2α (that acts as a vasoconstrictor and induces myometrial contraction) and prostaglandin E2 (acting as a vasodilator, leading to increased edema) in endometrium has important implications in menstruation (reviewed in Rizner, 2012). Significant increase in AKR1B1 immunoreactivity in a subset of stromal cells in the LS and M phase endometrial tissue has been reported (Catalano et al., 2011). Furthermore, in a very recent publication it was

shown that *AKR1B1* mRNA and protein levels were significantly higher in ovarian lesion samples of endometriosis patients compared to eutopic endometria of healthy women when secretory phase samples were compared but there were no differences between proliferative phase samples (Sinreih et al., 2015). A similar menstrual cycle-dependent expression difference was demonstrated in comparisons between eu- and ectopic endometria of the same individuals (Rakhila et al., 2013) but interestingly, this study found no differences between endometria of healthy women and endometriosis patients' eutopic or ectopic endometria. Although we did not see differential methylation of *AKR1B1* between menstrual cycle phases, cyclic epigenetic changes could still be present; however, as most of our specific subgroups were relatively small, it is likely that we were not able to detect all potential changes.

Also, one of the differentially methylated genes in our study was *AHRR*, which shows increased gene expression in fetal tissues exposed to environmental or even lower levels of bisphenol (Nishizawa et al., 2005). It has been proposed that developmental exposure to environmental toxins may induce irregular methylation patterns and thereby permanently alter the expression of *AHRR* (Aragon et al., 2008). *In utero* exposure to endocrine disruptor bisphenol could be one potential cause triggering the abnormal fetal endometrial cell migration into ectopic location, as mice exposed *in utero* to bisphenol exhibited an endometriosis-like phenotype (Signorile et al., 2010b).

Furthermore, our validation analysis confirmed differential methylation of CpG in *SLC43A3* promoter; however, only between the MS- phase patients and controls indicating influences both from disease and menstrual cycle phase. *SLC43A3* is a purine-selective nucleobase transporter (Furukawa et al., 2015) shown to be expressed during embryogenesis (Stuart et al., 2001) but its possible role in endometrium or endometriosis development remains to be elucidated.

Based on our results we propose that although the epigenetic modifications are probably not the primary source contributing to endometriosis development, the possible role of small-scale alterations in endometrium found in this study is intriguing and worth further studies.

6.3. Relevance of the findings in the context of molecular diagnostics

To date, there is no molecular test for endometriosis and histological verification of endometrial glands and/or stromal cells of biopsied samples is still needed to confirm the diagnosis of endometriosis. Although histological evaluation of biopsies is routinely used in everyday practise, a number of previous studies have demonstrated that almost half of the surgical specimens removed during laparoscopy are not confirmed in the following histological assessments (Moen et al., 1992; Stratton et al., 2003; Stegmann et al., 2008; Fernando et al., 2013) and some of the removed biopsies are lesion-like structures that include fibrosis, inflammatory changes and normal peritoneum (Wanyonyi et al., 2011). Besides

traditional HE staining-based histological evaluation, immunohistochemistry (IHC) analysis with antibody against cell-surface metallo-endopeptidase CD10 has been used to improve the recognition of ectopic stromal cells (Sumathi et al., 2002). Although the added value of CD10 IHC in case of uncertain diagnosis and atypical endometriosis has been confirmed and there is evidence that this will significantly increase the diagnostic rate when combined with HE staining (Potlog-Nahari et al., 2004) there are also possible limitations using CD10 for diagnostics. The major shortage is that the CD10 IHC does not detect only endometrial stroma but also lymphocytes and this could give false positive results in case of tissue samples with lymphoid infiltration. Therefore, for correct diagnosis, the visualisation of epithelial glandular structures using HE staining is still needed.

In Study II, we found that the expression signature of a subset of three miRNAs, miR-449a/miR-200a/miR-200b that are mostly expressed in endometrial epithelial cells, enables to separate endometriotic lesions from non-diseased tissues with a sensitivity of 95.8% and specificity of 95.5%, and therefore a molecular test based on these miRNAs offers an alternative to routine histological assessment. During the routine histological assessment, several tissue sections from different parts of the biopsy are examined and if the proportion of endometriotic cells in biopsied specimen is minute, the presence of endometrial stromal and epithelial cells may be missed. The advantage of a molecular miRNA-based test is that it would allow the evaluation of the entire biopsy at once giving accurate diagnosis with high sensitivity and specificity. Furthermore, there is no need for a highly experienced pathologist to perform and interpret the analysis.

However, it should be pointed out that although we found a well-performing miRNA molecular signature that could be of help in endometriosis diagnostics, the usability of this test as an independent diagnostic tool in everyday diagnostics is still too ambiguous and more markers confirming also the presence of endometrial stromal cells (such as CD10) and excluding other pathologic changes, should be added. Still, we believe that miRNA-based molecular diagnostic tests are very promising and would offer a good alternative to traditional histological assessment.

6.4. Study limitations

Some limitations of the studies presented in the current thesis should be acknowledged. As the cost of high-throughput technologies is relatively high, the sample sizes used in all our studies have been rather small, thus the studies are relatively underpowered to see small and subtle differences between investigated tissues. Furthermore, the variance of individual measurements has a larger effect in case of small study groups. It is known that different subtypes of endometriosis with diverse malignant potential exist (Oral et al., 2003); therefore, as Study I revealed no somatic alterations in lesions, we may assume

that none of our samples was a pre-malignant lesion. Although the prevalence of ovarian cancer arising from atypical ovarian endometriosis (ranging from 0.7% to 17%) is small (Grandi et al., 2015) and the malignant transformation of superficial peritoneal lesions is extremely rare (Marchand et al., 2013), we cannot exclude the possibility that some of the previous studies, reporting large number of chromosomal alterations, have studied pre-malignant samples. Also, in this study we used SNP microarray to determine genomic alterations and therefore it could be that some of the existing rare SCNAs might be missed as the probe distribution on a SNP array depends on the availability of informative SNPs throughout the genome.

In the miRNA sequencing study (Study II), only peritoneal endometriotic lesions were used to determine the discriminative power of the miRNA signature (miR-449a/miR-200a/miR-200b) and therefore, we cannot assure that the proposed molecular test is valid for other types of endometriosis (endometrioma, extra-genital endometriosis etc.). Although our endometrial DNA methylation study (Study III) was the largest that has been performed so far, the number of studied samples was still too small to obtain sufficient study power for detection of small scale DNA methylation changes between patients and controls. Furthermore, the size of some menstrual cycle phase groups remained relatively low and restricted the possibility to compare patients and controls from each phase separately. Also, histological endometrial dating was available only for healthy volunteers from MS group. It could be assumed that the self-reported menstrual cycle day is less accurate for menstrual cycle phase dating and it may have some negative impact on menstrual cycle phase-specific analysis.

6.5. Future perspectives

The results presented in this thesis suggest that analysis of entire biopsied specimens does not provide clear insight into the molecular changes occurring inside the lesions. To shed light on true changes without any contamination from adjacent tissues/cells, the pure populations of endometrial stromal, epithelial and stem cells from lesions are needed. Therefore, in my opinion the major challenge in future endometriosis research is not related to the research methodology or data analysis *per se*, as molecular technologies enabling analysis of single cells are already available, but are rather associated with difficulties related to obtaining specific single cells or cell populations from lesions. Previously, we have used LCM technique (Study I) for isolating endometrial epithelial and stromal cells from lesions but as DNA and RNA quality obtained by this methodology varies largely (Aaltonen et al., 2011; Cummings et al., 2011) this method would probably not be the first choice. In our recent study (Krjutskov et al., 2016) we used combinations of fluorescently labelled antibodies (CD13 and CD9) that are shown to be markers of endometrial stromal and epithelial cells (Kato et al., 2007) and FACS to isolate single cells or cell

populations from endometrial biopsies. We believe that this methodology can be successfully transferred to endometriotic lesion studies to obtain sufficient amounts of pure endometrial stromal and epithelial cells for down-stream analysis. Only biological data from pure cell populations from lesions could uncover the true molecular changes behind the disease and open the doors for new ideas for development of diagnostic methods and disease-specific treatment.

7. CONCLUSION

Based on the results, the following conclusions can be made:

1. No SCNAs were present in eutopic or ectopic endometria in our study group and molecular mechanisms other than chromosomal rearrangements most likely contribute to the initiation and progression of endometriosis.

2. The miRNA expression in surrounding peritoneal tissue masked most of the miRNA expression differences that could originate from a small proportion of endometrial cells inside the lesion and therefore only miRNAs with large expression differences can be seen.

3. All upregulated miRNAs (mirR-34c, miR-449a, miR-200a, miR-200b and miR-141) reflected the presence of endometrial epithelial cells in lesions and thus were good markers to discriminate true endometriotic lesions from lesion-like tissue structures. The expression signature of three miRNAs, miR-449a/miR-200a/miR-200b offers a good alternative to routine histological examination for the accurate assessment of endometriotic lesions.

4. Endometrial DNA methylation signature in women with and without endometriosis was highly similar but largely influenced by the menstrual cycle phase. Epigenetic modifications in endometrium are probably not the primary contributor to endometriosis development.

5. Normal epigenetic changes occurring in endometrium across the menstrual cycle phases should be considered when looking for disease-specific DNA methylation markers.

SUMMARY IN ESTONIAN

Endomeetriumi ja endometrioosikollete molekulaarse profiili iseloomustamine

Endometrioos on tõsine ja sage günekoloogiline haigus, mida iseloomustab endomeetriumi-laadse koe paiknemine ja toimimine väljaspool emakaõõnt. Sarnaselt normaalsele endomeetriumi koele, kus menstruaaltsükli jooksult toimub koe kasvamine ja irdumine, on ka kõhuõõnes paiknevad endometrioosikolded menstruaaltsüklist mõjutatud. Kõhuõõnes tsükliliselt veritsevad kolded kutsuvad esile põletikulisi ja liitelisi protsesse, mis põhjustavad alakõhuvalusid ning sageli ka viljatust. Arvatakse, et ligikaudu pooltel viljatutest naistest võib viljatus olla tingitud just endometrioosist. Endometrioosi diagnoosimiseks puuduvad siiani mitte-invasiivsed meetodid ning korrektne diagnoos põhineb laparoskoopilisel operatsioonil ja kollete histoloogilisel uuringul. Seetõttu on baasteaduslikud uuringud, mis aitaksid leida haigust põhjustavaid molekulaarseid muutusi nii endometrioosikolletes kui endomeetriumis, väga olulised.

Vaatamata ulatuslikele uuringutele ei ole siiani selge, kuidas endomeetriumi rakud kõhuõõnde satuvad, miks nad seal kinnituvad ja hakkavad kasvama vales kohas. Endometrioosi patogeneesimehhanismide uurimisele on oluliselt kaasa aidanud mikrokiibi- ja sekveneerimistehnoloogiate kiire areng, mis võimaldab korraga saada suurt hulka bioloogiliselt olulist informatsiooni. Tänaseks on teostatud ligikaudu 50 erinevat molekulaarse profiili määramise tööd, kus kirjeldatakse endomeetriumis ja endometrioosikolletes toimunud muutusi. Nii on välja toodud, et sarnaselt kasvajatele on ka endometrioosikoldeid moodustavates rakkudes toimunud somaatilised DNA koopiaarvu muutused. Samuti on endometrioosi kujunemisega seostatud muutusi lühikeste mittekodeerivate mikroRNA (miRNA) molekulide ekspressioonis, mis omakorda mõjutab valke kodeerivate geenide avaldumist transkriptsioonijärgselt. Hiljutised uuringud on välja toonud DNA metülatsioonimustri muutuste võimaliku rolli endometrioosi tekkes. Kuigi molekulaarse profiili määramise uuringud on näidanud muutusi endometrioosikollete DNAs, miRNA-de tasemetes ja metülatsiooniprofiilis, on erinevate uuringute tulemused siiski olnud sageli vastukäivad ning haiguse võimalikke patogeneesimehhanisme pole siiani suudetud selgitada. Varasemate uuringute tulemuste vähene kattuvus viitab suure tõenäosusega probleemidele katsete disainis ja seetõttu on väga oluline juba uuringut planeerides pöörata tähelepanu võimalikele kitsaskohtadele, mis võiksid saadud tulemusi mõjutada.

Uurimistöö eesmärgid

Käesoleva uurimistöö eesmärgiks oli tuvastada võimalikud endometrioosi kujunemist põhjustavad genoomsed muutused nii endometrioosikolletes kui ka endomeetriumis, kasutades selleks hoolikalt valitud katseskeemi ja mikrokiipidel ning süvasekveneerimisel põhinevaid tehnoloogiaid.

Sellest tulenevalt olid töö alameesmärgid järgmised:

1. Tuvastada *de novo* somaatiliste kromosomaalsete aberratsioonide olemasolu endometrioosikolletes ja endomeetriumis ning määrata nende võimalik olulisus endometrioosi patogeneesis.
2. Kirjeldada ja leida endometrioosikolletele iseloomulik miRNA-de profiil, mis aitaks välja selgitada haiguse võimalikke tekkepõhjuseid.
3. Selgitada välja DNA metülatsioonimustri erinevused tervetel ja endometrioosiga naiste endomeetriumites ning leida võimalikud haigusega seotud metülatsiooni markerid.

Metoodika

Käesolevas uuringus osalenud endometrioosiga naised värvati Tartu Ülikooli Kliinikumi naistekliinikust, Elite Kliinikust või John Radcliffe (Oxford, UK) haiglast. Kõik uuringus osalenud naised allkirjastasid uuringus osalemise nõusoleku vormi ja täitsid reproduktiivanamneesi käsitleva küsimustiku. Kontrollgrupi naiste vere- ja koeproovid pärinesid kas TÜ Geenivaramust, Nova Vita Kliinikust, Elite Kliinikust või John Radcliffe haigla patsientidelt. Kõik uuringus osalenud naised olid reproduktiivses eas ja ei olnud tarvitanud hormonaalseid preparaate uuringule eelnenud kolme kuu jooksul.

Somaatiliste DNA koopiaarvude tuvastamiseks kasutati 11 patsiendi endomeetriumi, vere ja laserpüüdur mikrodissektsiooni abil eraldatud endometrioosikolde DNAd, mida analüüsiti SNP-mikrokiipidega. Uuringus valideeriti üks DNA koopiaarvu muutus 187 patsiendi ja 171 kontroll-indiviidi vere DNAst, kasutades kvantitatiivset reaal-aja PCR meetodit (qRT-PCR). miRNA-de profiili tuvastamiseks süvasekveneeriti kahe naise endomeetriumist, endometrioosikolletest ja kollet ümbritsevast tervest koest pärinev RNA. Endometrioosikolletes kõrgemalt ekspresseerunud miRNA-de tasemed valideeriti endometrioosiga ja endometrioosita naiste koeproovides qRT-PCR meetodil. Endomeetriumi strooma ja epiteeli rakud eraldati fluorestsents-aktiveeritud läbivoolutsütomeetriga. DNA metülatsiooniprofiil määrati 31 endometrioosiga ja 24 endometrioosita naisel kasutades mikrokiipe. Uuringu tulemused valideeriti 15 endometrioosiga ja 14 endometrioosita naise endomeetriumi bisulfit meetodil töödeldud DNA Sanger sekveneerimise abil.

Tulemused ja järeldused

1. Endometrioosiga naiste endometrioosikolletes ja endomeetriumis ei ole toimunud suuremahulisi somaatilisi DNA koopiaarvu muutusi, mis võiksid olla seotud endometrioosi kujunemisega.
2. Kasutades endometrioosikollete uurimisel laparoskoopia käigus saadud biopsiaid, ei ole ümbritseva terve koe mõjude tõttu võimalik määrata kolletele iseloomulikku miRNA-de mustrit. Siiski suutsime tuvastada viis miRNA-t (mirR-34c, miR-449a, miR-200a, miR-200b ja miR-141), mille ekspressioon on kolde rakkudes tunduvalt kõrgem võrreldes kollet ümbritseva terve

koega. Kolde ja endomeetriumi koeproovide võrdlus näitas aga suurt erinevust, mis tulenes suuresti kollet ümbritseva koe mõjudest. Sellest saame järeldada, et haigusele iseloomulike miRNA tasemete muutuste tuvastamiseks tuleb arvesse võtta iga koetüübi normaalset miRNA profiili ja vältida endometrioosikolde võrdlemist endomeetriumiga, mis sisaldab suurel määral kudet, millel ta paikneb ja vaid vähesel hulgal endomeetriumi spetsiifilisi rakke.

3. Endometrioosikolletes kõrgemalt ekspresseeritud miRNA-d, (mirR-34c, miR-449a, miR-200a, miR-200b ja miR-141) peegeldasid eelkõige endomeetriumist pärineva epiteeli rakkude olemasolu kolletes. Kuna histoloogilise uuringu käigus ei suudeta alati endometrioosikoldeid kolde-laadsetest kudedest eristada, siis pakkusime välja parimat sensitiivsust ja spetsiifilisust näidanud kolme miRNA kombinatsioonil (miR-449a/miR-200a/miR-200b) põhineva molekulaarse testi endometrioosikolde tuvastamiseks ja endometrioosi diagnoosi kinnitamiseks. Väljapakutud endometrioosikolde molekulaarne test on hea alternatiiv praegu kasutuses olevale histoloogilisele uuringule.
4. Endomeetriumi DNA metülatsioonimuster endometrioosiga ja endometrioosita naistel oli väga sarnane ja seetõttu võib oletada, et endomeetriumi epigeneetilised muutused ei oma haiguse kujunemises peamist rolli.
5. Endomeetriumis toimuvad normaalsed epigeneetilised muutused kogu menstruaaltsükli jooksul ja seetõttu tuleb haiguseseoseliste DNA metülatsiooni-markerite otsimisel seda kindlasti arvestada.

REFERENCES

Aaltonen KE, Ebbesson A, Wigerup C, Hedenfalk I. Laser capture microdissection (LCM) and whole genome amplification (WGA) of DNA from normal breast tissue – optimization for genome wide array analyses. *BMC Res Notes* 2011; **4**:69.

Absenger Y, Hess-Stumpp H, Kreft B, Kratzschmar J, Haendler B, Schutze N, Regidor PA, Winterhager E. Cyr61, a deregulated gene in endometriosis. *Mol Hum Reprod* 2004; **10**:399–407.

Adachi S, Tajima A, Quan J, Haino K, Yoshihara K, Masuzaki H, Katabuchi H, Ikuma K, Suginami H, Nishida N, Kuwano R, Okazaki Y, Kawamura Y, Sasaki T, Tokunaga K, Inoue I, Tanaka K. Meta-analysis of genome-wide association scans for genetic susceptibility to endometriosis in Japanese population. *J Hum Genet* 2010; **55**:816–821.

Aghajanova L, Giudice LC. Molecular evidence for differences in endometrium in severe versus mild endometriosis. *Reprod Sci* 2011; **18**:229–251.

Albertsen HM, Chettier R, Farrington P, Ward K. Genome-wide association study link novel loci to endometriosis. *PLoS One* 2013; **8**:e58257.

Altmae S, Esteban FJ, Stavreus-Evers A, Simon C, Giudice L, Lessey BA, Horcajadas JA, Macklon NS, D'Hooghe T, Campoy C, Fauser BC, Salamonsen LA, Salumets A. Guidelines for the design, analysis and interpretation of 'omics' data: focus on human endometrium. *Hum Reprod Update* 2014; **20**:12–28.

Anaf V, Simon P, Fayt I, Noel J. Smooth muscles are frequent components of endometriotic lesions. *Hum Reprod* 2000; **15**:767–771.

Aragon AC, Kopf PG, Campen MJ, Huwe JK, Walker MK. In utero and lactational 2,3,7,8-tetrachlorodibenzo-p-dioxin exposure: effects on fetal and adult cardiac gene expression and adult cardiac and renal morphology. *Toxicol Sci* 2008; **101**:321–330.

Arimoto T, Katagiri T, Oda K, Tsunoda T, Yasugi T, Osuga Y, Yoshikawa H, Nishii O, Yano T, Taketani Y, Nakamura Y. Genome-wide cDNA microarray analysis of gene-expression profiles involved in ovarian endometriosis. *Int J Oncol* 2003; **22**:551–560.

Aryee MJ, Jaffe AE, Corrada-Bravo H, Ladd-Acosta C, Feinberg AP, Hansen KD, Irizarry RA. Minfi: a flexible and comprehensive Bioconductor package for the analysis of Infinium DNA methylation microarrays. *Bioinformatics* 2014; **30**:1363–1369.

ASRM. Revised American Society for Reproductive Medicine classification of endometriosis: 1996. *Fertil Steril* 1997; **67**:817–821.

Banno K, Yanokura M, Kisu I, Yamagami W, Susumu N, Aoki D. MicroRNAs in endometrial cancer. *Int J Clin Oncol* 2013; **18**:186–192.

Barcena de Arellano ML, Mechsner S. The peritoneum – an important factor for pathogenesis and pain generation in endometriosis. *J Mol Med (Berl)* 2014; **92**:595–602.

Becker CM, Laufer MR, Stratton P, Hummelshoj L, Missmer SA, Zondervan KT, Adamson GD. World Endometriosis Research Foundation Endometriosis Phenome and Biobanking Harmonisation Project: I. Surgical phenotype data collection in endometriosis research. *Fertil Steril* 2014; **102**:1211–1222.

Beroukhim R, Mermel CH, Porter D, Wei G, Raychaudhuri S, Donovan J, Barretina J, Boehm JS, Dobson J, Urashima M et al.,. The landscape of somatic copy-number alteration across human cancers. *Nature* 2010; **463**:899–905.

Bird A. DNA methylation patterns and epigenetic memory. *Genes Dev* 2002; **16**:6–21.

Borghese B, Barbaux S, Mondon F, Santulli P, Pierre G, Vinci G, Chapron C, Vaiman D. Research resource: genome-wide profiling of methylated promoters in endometriosis reveals a subtelomeric location of hypermethylation. *Mol Endocrinol* 2010; **24**:1872–1885.

Brabletz S, Brabletz T. The ZEB/miR-200 feedback loop – a motor of cellular plasticity in development and cancer? *EMBO Rep* 2010; **11**:670–677.

Bracken CP, Gregory PA, Kolesnikoff N, Bert AG, Wang J, Shannon MF, Goodall GJ. A double-negative feedback loop between ZEB1-SIP1 and the microRNA-200 family regulates epithelial-mesenchymal transition. *Cancer Res* 2008; **68**:7846–7854.

Braza-Boils A, Mari-Alexandre J, Gilabert J, Sanchez-Izquierdo D, Espana F, Estelles A, Gilabert-Estelles J. MicroRNA expression profile in endometriosis: its relation to angiogenesis and fibrinolytic factors. *Hum Reprod* 2014; **29**:978–988.

Burney RO, Giudice LC. Pathogenesis and pathophysiology of endometriosis. *Fertil Steril* 2012; **98**:511–519.

Burney RO, Hamilton AE, Aghajanova L, Vo KC, Nezhat CN, Lessey BA, Giudice LC. MicroRNA expression profiling of eutopic secretory endometrium in women with versus without endometriosis. *Mol Hum Reprod* 2009; **15**:625–631.

Burney RO, Talbi S, Hamilton AE, Vo KC, Nyegaard M, Nezhat CR, Lessey BA, Giudice LC. Gene expression analysis of endometrium reveals progesterone resistance and candidate susceptibility genes in women with endometriosis. *Endocrinology* 2007; **148**:3814–3826.

Canis M, Bourdel N, Houlle C, Gremeau AS, Botchorishvili R, Matsuzaki S. Endometriosis may not be a chronic disease: an alternative theory offering more optimistic prospects for our patients. *Fertil Steril* 2016; **105**:32–34.

Carson DD, Lagow E, Thathiah A, Al-Shami R, Farach-Carson MC, Vernon M, Yuan L, Fritz MA, Lessey B. Changes in gene expression during the early to mid-luteal (receptive phase) transition in human endometrium detected by high-density microarray screening. *Mol Hum Reprod* 2002; **8**:871–879.

Catalano RD, Wilson MR, Boddy SC, Jabbour HN. Comprehensive expression analysis of prostanoid enzymes and receptors in the human endometrium across the menstrual cycle. *Mol Hum Reprod* 2011; **17**:182–192.

Chettier R, Ward K, Albertsen HM. Endometriosis is associated with rare copy number variants. *PLoS One* 2014; **9**:e103968.

Colella S, Yau C, Taylor JM, Mirza G, Butler H, Clouston P, Bassett AS, Seller A, Holmes CC, Ragoussis J. QuantiSNP: an Objective Bayes Hidden-Markov Model to detect and accurately map copy number variation using SNP genotyping data. *Nucleic Acids Res* 2007; **35**:2013–2025.

Cramer DW, Missmer SA. The epidemiology of endometriosis. *Ann N Y Acad Sci* 2002; **955**:11–22; discussion 34–16, 396–406.

Creighton CJ, Benham AL, Zhu H, Khan MF, Reid JG, Nagaraja AK, Fountain MD, Dziadek O, Han D, Ma L, Kim J, Hawkins SM, Anderson ML, Matzuk MM, Gunaratne PH. Discovery of novel microRNAs in female reproductive tract using next generation sequencing. *PLoS One* 2010; **5**:e9637.

Crispi S, Piccolo MT, D'Avino A, Donizetti A, Viceconte R, Spyrou M, Calogero RA, Baldi A, Signorile PG. Transcriptional profiling of endometriosis tissues identifies genes related to organogenesis defects. *J Cell Physiol* 2013; **228**:1927–1934.

Cummings M, McGinley CV, Wilkinson N, Field SL, Duffy SR, Orsi NM. A robust RNA integrity-preserving staining protocol for laser capture microdissection of endometrial cancer tissue. *Anal Biochem* 2011; **416**:123–125.

D'Hooghe TM, Bambra CS, Cornillie FJ, Isahakia M, Koninckx PR. Prevalence and laparoscopic appearance of spontaneous endometriosis in the baboon (Papio anubis, Papio cynocephalus). *Biol Reprod* 1991; **45**:411–416.

Diaz-Gimeno P, Horcajadas JA, Martinez-Conejero JA, Esteban FJ, Alama P, Pellicer A, Simon C. A genomic diagnostic tool for human endometrial receptivity based on the transcriptomic signature. *Fertil Steril* 2011; **95**:50–60, 60 e51–15.

Dunselman GA, Vermeulen N, Becker C, Calhaz-Jorge C, D'Hooghe T, De Bie B, Heikinheimo O, Horne AW, Kiesel L, Nap A, Prentice A, Saridogan E, Soriano D, Nelen W. ESHRE guideline: management of women with endometriosis. *Hum Reprod* 2014; **29**:400–412.

Dyson MT, Roqueiro D, Monsivais D, Ercan CM, Pavone ME, Brooks DC, Kakinuma T, Ono M, Jafari N, Dai Y, Bulun SE. Genome-wide DNA methylation analysis predicts an epigenetic switch for GATA factor expression in endometriosis. *PLoS Genet* 2014; **10**:e1004158.

Eggers JC, Martino V, Reinbold R, Schafer SD, Kiesel L, Starzinski-Powitz A, Schuring AN, Kemper B, Greve B, Gotte M. microRNA miR-200b affects proliferation, invasiveness and stemness of endometriotic cells by targeting ZEB1, ZEB2 and KLF4. *Reprod Biomed Online* 2016;

Eyster KM, Klinkova O, Kennedy V, Hansen KA. Whole genome deoxyribonucleic acid microarray analysis of gene expression in ectopic versus eutopic endometrium. *Fertil Steril* 2007; **88**:1505–1533.

Fassbender A, Rahmioglu N, Vitonis AF, Vigano P, Giudice LC, D'Hooghe TM, Hummelshoj L, Adamson GD, Becker CM, Missmer SA, Zondervan KT. World Endometriosis Research Foundation Endometriosis Phenome and Biobanking Harmonisation Project: IV. Tissue collection, processing, and storage in endometriosis research. *Fertil Steril* 2014; **10**:1244–1253.

Fassbender A, Verbeeck N, Bornigen D, Kyama CM, Bokor A, Vodolazkaia A, Peeraer K, Tomassetti C, Meuleman C, Gevaert O, Van de Plas R, Ojeda F, De Moor B, Moreau Y, Waelkens E, D'Hooghe TM. Combined mRNA microarray and proteomic analysis of eutopic endometrium of women with and without endometriosis. *Hum Reprod* 2012; **27**:2020–2029.

Fayez JA, Vogel MF. Comparison of different treatment methods of endometriomas by laparoscopy. *Obstet Gynecol* 1991; **78**:660–665.

Fernando S, Soh PQ, Cooper M, Evans S, Reid G, Tsaltas J, Rombauts L. Reliability of visual diagnosis of endometriosis. *J Minim Invasive Gynecol* 2013; **20**:783–789.

Filigheddu N, Gregnanin I, Porporato PE, Surico D, Perego B, Galli L, Patrignani C, Graziani A, Surico N. Differential expression of microRNAs between eutopic and ectopic endometrium in ovarian endometriosis. *J Biomed Biotechnol* 2010; **2010**:369549.

Furukawa J, Inoue K, Maeda J, Yasujima T, Ohta K, Kanai Y, Takada T, Matsuo H, Yuasa H. Functional identification of SLC43A3 as an equilibrative nucleobase transporter involved in purine salvage in mammals. *Sci Rep* 2015; **5**:15057.

Gilabert-Estelles J, Braza-Boils A, Ramon LA, Zorio E, Medina P, Espana F, Estelles A. Role of microRNAs in gynecological pathology. *Curr Med Chem* 2012; **19**:2406–2413.

Giudice LC. Clinical practice. Endometriosis. *N Engl J Med* 2010; **362**:2389–2398.

Gogusev J, Bouquet de Joliniere J, Telvi L, Doussau M, du Manoir S, Stojkoski A, Levardon M. Detection of DNA copy number changes in human endometriosis by comparative genomic hybridization. *Hum Genet* 1999; **105**:444–451.

Grandi G, Toss A, Cortesi L, Botticelli L, Volpe A, Cagnacci A. The Association between Endometriomas and Ovarian Cancer: Preventive Effect of Inhibiting Ovulation and Menstruation during Reproductive Life. *Biomed Res Int* 2015; **2015**:751571.

Guo SW, Wu Y, Strawn E, Basir Z, Wang Y, Halverson G, Montgomery K, Kajdacsy-Balla A. Genomic alterations in the endometrium may be a proximate cause for endometriosis. *Eur J Obstet Gynecol Reprod Biol* 2004; **116**:89–99.

Haouzi D, Assou S, Mahmoud K, Tondeur S, Reme T, Hedon B, De Vos J, Hamamah S. Gene expression profile of human endometrial receptivity: comparison between natural and stimulated cycles for the same patients. *Hum Reprod* 2009; **24**:1436–1445.

Hawkins SM, Creighton CJ, Han DY, Zariff A, Anderson ML, Gunaratne PH, Matzuk MM. Functional microRNA involved in endometriosis. *Mol Endocrinol* 2011; **25**:821–832.

Heberle H, Meirelles GV, da Silva FR, Telles GP, Minghim R. InteractiVenn: a web-based tool for the analysis of sets through Venn diagrams. *BMC Bioinformatics* 2015; **16**:169.

Hever A, Roth RB, Hevezi P, Marin ME, Acosta JA, Acosta H, Rojas J, Herrera R, Grigoriadis D, White E, Conlon PJ, Maki RA, Zlotnik A. Human endometriosis is associated with plasma cells and overexpression of B lymphocyte stimulator. *Proc Natl Acad Sci U S A* 2007; **104**:12451–12456.

Hill L, Browne G, Tulchinsky E. ZEB/miR-200 feedback loop: at the crossroads of signal transduction in cancer. *Int J Cancer* 2013; **132**:745–754.

Houshdaran S, Zelenko Z, Irwin JC, Giudice LC. Human endometrial DNA methylome is cycle-dependent and is associated with gene expression regulation. *Mol Endocrinol* 2014; **28**:1118–1135.

Hsu AL, Khachikyan I, Stratton P. Invasive and noninvasive methods for the diagnosis of endometriosis. *Clin Obstet Gynecol* 2010; **53**:413–419.

Hu S, Yao G, Wang Y, Xu H, Ji X, He Y, Zhu Q, Chen Z, Sun Y. Transcriptomic changes during the pre-receptive to receptive transition in human endometrium detected by RNA-Seq. *J Clin Endocrinol Metab* 2014; **99**:E2744–2753.

Hu WP, Tay SK, Zhao Y. Endometriosis-specific genes identified by real-time reverse transcription-polymerase chain reaction expression profiling of endometriosis versus autologous uterine endometrium. *J Clin Endocrinol Metab* 2006; **91**:228–238.

Kao LC, Germeyer A, Tulac S, Lobo S, Yang JP, Taylor RN, Osteen K, Lessey BA, Giudice LC. Expression profiling of endometrium from women with endometriosis reveals candidate genes for disease-based implantation failure and infertility. *Endocrinology* 2003; **144**:2870–2881.

Karhu R, Kahkonen M, Kuukasjarvi T, Pennanen S, Tirkkonen M, Kallioniemi O. Quality control of CGH: impact of metaphase chromosomes and the dynamic range of hybridization. *Cytometry* 1997; **28**:198–205.

Kato K, Yoshimoto M, Adachi S, Yamayoshi A, Arima T, Asanoma K, Kyo S, Nakahata T, Wake N. Characterization of side-population cells in human normal endometrium. *Hum Reprod* 2007; **22**:1214–1223.

Kennedy S, Bergqvist A, Chapron C, D'Hooghe T, Dunselman G, Greb R, Hummelshoj L, Prentice A, Saridogan E. ESHRE guideline for the diagnosis and treatment of endometriosis. *Hum Reprod* 2005; **20**:2698–2704.

Khan MA, Sengupta J, Mittal S, Ghosh D. Genome-wide expressions in autologous eutopic and ectopic endometrium of fertile women with endometriosis. *Reprod Biol Endocrinol* 2012; **10**:84.

Kobayashi H, Yamashita Y, Iwase A, Yoshikawa Y, Yasui H, Kawai Y, Uchida K, Uno N, Akatsuka S, Takahashi T, Kikkawa F, Toyokuni S. The ferroimmunomodulatory role of ectopic endometriotic stromal cells in ovarian endometriosis. *Fertil Steril* 2012; **98**:415–422 e411–412.

Koninckx PR, Kennedy SH, Barlow DH. Endometriotic disease: the role of peritoneal fluid. *Hum Reprod Update* 1998; **4**:741–751.

Konno R, Yamada-Okabe H, Fujiwara H, Uchiide I, Shibahara H, Ohwada M, Ihara T, Sugamata M, Suzuki M. Role of immunoreactions and mast cells in pathogenesis of human endometriosis – morphologic study and gene expression analysis. *Hum Cell* 2003; **16**:141–149.

Korpal M, Kang Y. The emerging role of miR-200 family of microRNAs in epithelial-mesenchymal transition and cancer metastasis. *RNA Biol* 2008; **5**:115–119.

Krjutskov K, Katayama S, Saare M, Vera-Rodriguez M, Lubenets D, Samuel K, Laisk-Podar T, Teder H, Einarsdottir E, Salumets A, Kere J. Single-cell transcriptome analysis of endometrial tissue. *Hum Reprod* 2016;

Laudanski P, Charkiewicz R, Kuzmicki M, Szamatowicz J, Charkiewicz A, Niklinski J. MicroRNAs expression profiling of eutopic proliferative endometrium in women with ovarian endometriosis. *Reprod Biol Endocrinol* 2013; **11**:78.

Laudanski P, Charkiewicz R, Tolwinska A, Szamatowicz J, Charkiewicz A, Niklinski J. Profiling of Selected MicroRNAs in Proliferative Eutopic Endometrium of Women with Ovarian Endometriosis. *Biomed Res Int* 2015; **2015**:760698.

Lebovic DI, Baldocchi RA, Mueller MD, Taylor RN. Altered expression of a cell-cycle suppressor gene, Tob-1, in endometriotic cells by cDNA array analyses. *Fertil Steril* 2002; **78**:849–854.

Li LC, Dahiya R. MethPrimer: designing primers for methylation PCRs. *Bioinformatics* 2002; **18**:1427–1431.

Li W, Olivier M. Current analysis platforms and methods for detecting copy number variation. *Physiol Genomics* 2013; **45**:1–16.

Li X, Zhang Y, Zhao L, Wang L, Wu Z, Mei Q, Nie J, Li Y, Fu X, Wang X, Meng Y, Han W. Whole-exome sequencing of endometriosis identifies frequent alterations in genes involved in cell adhesion and chromatin-remodeling complexes. *Hum Mol Genet* 2014; **23**:6008–6021.

Lize M, Klimke A, Dobbelstein M. MicroRNA-449 in cell fate determination. *Cell Cycle* 2011; **10**:2874–2882.

Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. *Methods* 2001; **25**:402–408.

Lokk K, Modhukur V, Rajashekar B, Martens K, Magi R, Kolde R, Koltsina M, Nilsson TK, Vilo J, Salumets A, Tonisson N. DNA methylome profiling of human tissues identifies global and tissue-specific methylation patterns. *Genome Biol* 2014; **15**:r54.

Ludwig H, Spornitz UM. Microarchitecture of the human endometrium by scanning electron microscopy: menstrual desquamation and remodeling. *Ann N Y Acad Sci* 1991; **622**:28–46.

Macer ML, Taylor HS. Endometriosis and infertility: a review of the pathogenesis and treatment of endometriosis-associated infertility. *Obstet Gynecol Clin North Am* 2012; **39**:535–549.

Marchand E, Hequet D, Thoury A, Barranger E. Malignant transformation of superficial peritoneal endometriosis lesion. *BMJ Case Rep* 2013; **2013**:007730.

Mazur K (2005) Diagnosis of Endometrial Biopsies and Curettings. A practical Approach. Second edn. Springer,

Matsuzaki S, Canis M, Vaurs-Barriere C, Boespflug-Tanguy O, Dastugue B, Mage G. DNA microarray analysis of gene expression in eutopic endometrium from patients with deep endometriosis using laser capture microdissection. *Fertil Steril* 2005; **84 Suppl 2**:1180–1190.

Matsuzaki S, Canis M, Vaurs-Barriere C, Pouly JL, Boespflug-Tanguy O, Penault-Llorca F, Dechelotte P, Dastugue B, Okamura K, Mage G. DNA microarray analysis of gene expression profiles in deep endometriosis using laser capture micro-dissection. *Mol Hum Reprod* 2004; **10**:719–728.

May KE, Conduit-Hulbert SA, Villar J, Kirtley S, Kennedy SH, Becker CM. Peripheral biomarkers of endometriosis: a systematic review. *Hum Reprod Update* 2010; **16**:651–674.

Mercer TR, Mattick JS. Structure and function of long noncoding RNAs in epigenetic regulation. *Nat Struct Mol Biol* 2013; **20**:300–307.

Moen MH, Halvorsen TB. Histologic confirmation of endometriosis in different peritoneal lesions. *Acta Obstet Gynecol Scand* 1992; **71**:337–342.

Morris AP, Voight BF, Teslovich TM, Ferreira T, Segre AV, Steinthorsdottir V, Strawbridge RJ, Khan H, Grallert H, Mahajan A et al.,. Large-scale association analysis provides insights into the genetic architecture and pathophysiology of type 2 diabetes. *Nat Genet* 2012; **44**:981–990.

Muzii L, Bianchi A, Bellati F, Cristi E, Pernice M, Zullo MA, Angioli R, Panici PB. Histologic analysis of endometriomas: what the surgeon needs to know. *Fertil Steril* 2007; **87**:362–366.

Naqvi H, Ilagan Y, Krikun G, Taylor HS. Altered Genome-Wide Methylation in Endometriosis. *Reprod Sci* 2014; 1237–1243.

Nasu K, Kawano Y, Tsukamoto Y, Takano M, Takai N, Li H, Furukawa Y, Abe W, Moriyama M, Narahara H. Aberrant DNA methylation status of endometriosis: epigenetics as the pathogenesis, biomarker and therapeutic target. *J Obstet Gynaecol Res* 2011; **37**:683–695.

Nishizawa H, Imanishi S, Manabe N. Effects of exposure in utero to bisphenol a on the expression of aryl hydrocarbon receptor, related factors, and xenobiotic metabolizing enzymes in murine embryos. *J Reprod Dev* 2005; **51**:593–605.

Nisolle M, Donnez J. Peritoneal endometriosis, ovarian endometriosis, and adenomyotic nodules of the rectovaginal septum are three different entities. *Fertil Steril* 1997; **68**:585–596.

Nyholt DR, Low SK, Anderson CA, Painter JN, Uno S, Morris AP, MacGregor S, Gordon SD, Henders AK, Martin NG, Attia J, Holliday EG, McEvoy M, Scott RJ, Kennedy SH, Treloar SA, Missmer SA, Adachi S, Tanaka K, Nakamura Y, Zondervan KT, Zembutsu H, Montgomery GW. Genome-wide association meta-analysis identifies new endometriosis risk loci. *Nat Genet* 2012; **44**:1355–1359.

Ohlsson Teague EM, Van der Hoek KH, Van der Hoek MB, Perry N, Wagaarachchi P, Robertson SA, Print CG, Hull LM. MicroRNA-regulated pathways associated with endometriosis. *Mol Endocrinol* 2009; **23**:265–275.

Oral E, Ilvan S, Tustas E, Korbeyli B, Bese T, Demirkiran F, Arvas M, Kosebay D. Prevalence of endometriosis in malignant epithelial ovary tumours. *Eur J Obstet Gynecol Reprod Biol* 2003; **109**:97–101.

Ozdemir V, Suarez-Kurtz G, Stenne R, Somogyi AA, Someya T, Kayaalp SO, Kolker E. Risk assessment and communication tools for genotype associations with multifactorial phenotypes: the concept of "edge effect" and cultivating an ethical bridge between omics innovations and society. *Omics* 2009; **13**:43–61.

Pagliardini L, Gentilini D, Vigano P, Panina-Bordignon P, Busacca M, Candiani M, Di Blasio AM. An Italian association study and meta-analysis with previous GWAS confirm WNT4, CDKN2BAS and FN1 as the first identified susceptibility loci for endometriosis. *J Med Genet* 2013; **50**:43–46.

Painter JN, Anderson CA, Nyholt DR, Macgregor S, Lin J, Lee SH, Lambert A, Zhao ZZ, Roseman F, Guo Q, Gordon SD, Wallace L, Henders AK, Visscher PM, Kraft P, Martin NG, Morris AP, Treloar SA, Kennedy SH, Missmer SA, Montgomery GW, Zondervan KT. Genome-wide association study identifies a locus at 7p15.2 associated with endometriosis. *Nat Genet* 2011; **43**:51–54.

Park SM, Gaur AB, Lengyel E, Peter ME. The miR-200 family determines the epithelial phenotype of cancer cells by targeting the E-cadherin repressors ZEB1 and ZEB2. *Genes Dev* 2008; **22**:894–907.

Parrish RR, Day JJ, Lubin FD. Direct bisulfite sequencing for examination of DNA methylation with gene and nucleotide resolution from brain tissues. *Curr Protoc Neurosci* 2012; **Chapter 7**:Unit 7 24.

Pinto D, Marshall C, Feuk L, Scherer SW. Copy-number variation in control population cohorts. *Hum Mol Genet* 2007; **16 Spec No. 2**:R168–173.

Ponnampalam AP, Weston GC, Trajstman AC, Susil B, Rogers PA. Molecular classification of human endometrial cycle stages by transcriptional profiling. *Mol Hum Reprod* 2004; **10**:879–893.

Potlog-Nahari C, Feldman AL, Stratton P, Koziol DE, Segars J, Merino MJ, Nieman LK. CD10 immunohistochemical staining enhances the histological detection of endometriosis. *Fertil Steril* 2004; **82**:86–92.

Rahmioglu N, Fassbender A, Vitonis AF, Tworoger SS, Hummelshoj L, D'Hooghe TM, Adamson GD, Giudice LC, Becker CM, Zondervan KT, Missmer SA. World Endometriosis Research Foundation Endometriosis Phenome and Biobanking Harmonization Project: III. Fluid biospecimen collection, processing, and storage in endometriosis research. *Fertil Steril* 2014a;

Rahmioglu N, Nyholt DR, Morris AP, Missmer SA, Montgomery GW, Zondervan KT. Genetic variants underlying risk of endometriosis: insights from meta-analysis of eight genome-wide association and replication datasets. *Hum Reprod Update* 2014b; **20**:702–716.

Rakhila H, Carli C, Daris M, Lemyre M, Leboeuf M, Akoum A. Identification of multiple and distinct defects in prostaglandin biosynthetic pathways in eutopic and ectopic endometrium of women with endometriosis. *Fertil Steril* 2013; **100**:1650–1659 e1651–1652.

Rizner TL. Enzymes of the AKR1B and AKR1C Subfamilies and Uterine Diseases. *Front Pharmacol* 2012; **3**:34.

Robinson MD, McCarthy DJ, Smyth GK. edgeR: a Bioconductor package for differential expression analysis of digital gene expression data. *Bioinformatics* 2010; **26**:139–140.

Rogers PA, D'Hooghe TM, Fazleabas A, Giudice LC, Montgomery GW, Petraglia F, Taylor RN. Defining future directions for endometriosis research: workshop report from the 2011 World Congress of Endometriosis In Montpellier, France. *Reprod Sci* 2013; **20**:483–499.

Romero-Perez L, Lopez-Garcia MA, Diaz-Martin J, Biscuola M, Castilla MA, Tafe LJ, Garg K, Oliva E, Matias-Guiu X, Soslow RA, Palacios J. ZEB1 overexpression associated with E-cadherin and microRNA-200 downregulation is characteristic of undifferentiated endometrial carcinoma. *Mod Pathol* 2013;

Sampson JA. Metastatic or Embolic Endometriosis, due to the Menstrual Dissemination of Endometrial Tissue into the Venous Circulation. *Am J Pathol* 1927; **3**:93–110 143.

Sanfilippo JS, Wakim NG, Schikler KN, Yussman MA. Endometriosis in association with uterine anomaly. *Am J Obstet Gynecol* 1986; **154**:39–43.

Sapkota Y, Fassbender A, Bowdler L, Fung JN, Peterse D, O D, Montgomery GW, Nyholt DR, D'Hooghe TM. Independent Replication and Meta-Analysis for Endometriosis Risk Loci. *Twin Res Hum Genet* 2015; 1–8.

Sasson IE, Taylor HS. Stem cells and the pathogenesis of endometriosis. *Ann N Y Acad Sci* 2008; **1127**:106–115.

Sebat J, Lakshmi B, Troge J, Alexander J, Young J, Lundin P, Maner S, Massa H, Walker M, Chi M, Navin N, Lucito R, Healy J, Hicks J, Ye K, Reiner A, Gilliam TC, Trask B, Patterson N, Zetterberg A, Wigler M. Large-scale copy number polymorphism in the human genome. *Science* 2004; **305**:525–528.

Sha AG, Liu JL, Jiang XM, Ren JZ, Ma CH, Lei W, Su RW, Yang ZM. Genome-wide identification of micro-ribonucleic acids associated with human endometrial receptivity in natural and stimulated cycles by deep sequencing. *Fertil Steril* 2011; **96**:150–155 e155.

Sherwin JR, Sharkey AM, Mihalyi A, Simsa P, Catalano RD, D'Hooghe TM. Global gene analysis of late secretory phase, eutopic endometrium does not provide the basis for a minimally invasive test of endometriosis. *Hum Reprod* 2008; **23**:1063–1068.

Shi XY, Gu L, Chen J, Guo XR, Shi YL. Downregulation of miR-183 inhibits apoptosis and enhances the invasive potential of endometrial stromal cells in endometriosis. *Int J Mol Med* 2014; **33**:59–67.

Signorile PG, Baldi A. Endometriosis: new concepts in the pathogenesis. *Int J Biochem Cell Biol* 2010a; **42**:778–780.

Signorile PG, Spugnini EP, Mita L, Mellone P, D'Avino A, Bianco M, Diano N, Caputo L, Rea F, Viceconte R, Portaccio M, Viggiano E, Citro G, Pierantoni R, Sica V, Vincenzi B, Mita DG, Baldi F, Baldi A. Pre-natal exposure of mice to bisphenol A elicits an endometriosis-like phenotype in female offspring. *Gen Comp Endocrinol* 2010b; **168**:318–325.

Silveira CG, Abrao MS, Dias JA, Jr., Coudry RA, Soares FA, Drigo SA, Domingues MA, Rogatto SR. Common chromosomal imbalances and stemness-related protein expression markers in endometriotic lesions from different anatomical sites: the potential role of stem cells. *Hum Reprod* 2012; **27**:3187–3197.

Sinreih M, Anko M, Kene NH, Kocbek V, Rizner TL. Expression of AKR1B1, AKR1C3 and other genes of prostaglandin F2alpha biosynthesis and action in ovarian endometriosis tissue and in model cell lines. *Chem Biol Interact* 2015; **234**:320–331.

Stankiewicz P, Lupski JR. Structural variation in the human genome and its role in disease. *Annu Rev Med* 2010; **61**:437–455.

Stegmann BJ, Sinaii N, Liu S, Segars J, Merino M, Nieman LK, Stratton P. Using location, color, size, and depth to characterize and identify endometriosis lesions in a cohort of 133 women. *Fertil Steril* 2008; **89**:1632–1636.

Stranger BE, Forrest MS, Dunning M, Ingle CE, Beazley C, Thorne N, Redon R, Bird CP, de Grassi A, Lee C, Tyler-Smith C, Carter N, Scherer SW, Tavare S, Deloukas P, Hurles ME, Dermitzakis ET. Relative impact of nucleotide and copy number variation on gene expression phenotypes. *Science* 2007; **315**:848–853.

Stratton P, Winkel C, Premkumar A, Chow C, Wilson J, Hearns-Stokes R, Heo S, Merino M, Nieman LK. Diagnostic accuracy of laparoscopy, magnetic resonance imaging, and histopathologic examination for the detection of endometriosis. *Fertil Steril* 2003; **79**:1078–1085.

Stuart RO, Pavlova A, Beier D, Li Z, Krijanovski Y, Nigam SK. EEG1, a putative transporter expressed during epithelial organogenesis: comparison with embryonic transporter expression during nephrogenesis. *Am J Physiol Renal Physiol* 2001; **281**:F1148–1156.

Sumathi VP, McCluggage WG. CD10 is useful in demonstrating endometrial stroma at ectopic sites and in confirming a diagnosis of endometriosis. *J Clin Pathol* 2002; **55**:391–392.

Sun PR, Jia SZ, Lin H, Leng JH, Lang JH. Genome-wide profiling of long noncoding ribonucleic acid expression patterns in ovarian endometriosis by microarray. *Fertil Steril* 2014; **101**:1038–1046 e1037.

Sundqvist J, Xu H, Vodolazkaia A, Fassbender A, Kyama C, Bokor A, Gemzell-Danielsson K, D'Hooghe TM, Falconer H. Replication of endometriosis-associated single-nucleotide polymorphisms from genome-wide association studies in a Caucasian population. *Hum Reprod* 2013; **28**:835–839.

Zafrakas M, Tarlatzis BC, Streichert T, Pournaropoulos F, Wolfle U, Smeets SJ, Wittek B, Grimbizis G, Brakenhoff RH, Pantel K, Bontis J, Gunes C. Genome-wide micro-array gene expression, array-CGH analysis, and telomerase activity in advanced ovarian endometriosis: a high degree of differentiation rather than malignant potential. *Int J Mol Med* 2008; **21**:335–344.

Zhang B, Zhou Y, Lin N, Lowdon RF, Hong C, Nagarajan RP, Cheng JB, Li D, Stevens M, Lee HJ et al.,. Functional DNA methylation differences between tissues, cell types, and across individuals discovered using the M&M algorithm. *Genome Res* 2013; **23**:1522–1540.

Talbi S, Hamilton AE, Vo KC, Tulac S, Overgaard MT, Dosiou C, Le Shay N, Nezhat CN, Kempson R, Lessey BA, Nayak NR, Giudice LC. Molecular phenotyping of human endometrium distinguishes menstrual cycle phases and underlying biological processes in normo-ovulatory women. *Endocrinology* 2006; **147**:1097–1121.

Talseth-Palmer BA, Bowden NA, Hill A, Meldrum C, Scott RJ. Whole genome amplification and its impact on CGH array profiles. *BMC Res Notes* 2008; **1**:56.

Tamaresis JS, Irwin JC, Goldfien GA, Rabban JT, Burney RO, Nezhat C, DePaolo LV, Giudice LC. Molecular Classification of Endometriosis and Disease Stage Using High-Dimensional Genomic Data. *Endocrinology* 2014; en20141490.

Teo SM, Ku CS, Naidoo N, Hall P, Chia KS, Salim A, Pawitan Y. A population-based study of copy number variants and regions of homozygosity in healthy Swedish individuals. *J Hum Genet* 2011; **56**:524–533.

Torres A, Torres K, Wdowiak P, Paszkowski T, Maciejewski R. Selection and validation of endogenous controls for microRNA expression studies in endometrioid endometrial cancer tissues. *Gynecol Oncol* 2013; **130**:588–594.

Uno S, Zembutsu H, Hirasawa A, Takahashi A, Kubo M, Akahane T, Aoki D, Kamatani N, Hirata K, Nakamura Y. A genome-wide association study identifies genetic variants in the CDKN2BAS locus associated with endometriosis in Japanese. *Nat Genet* 2010; **42**:707–710.

Walter AJ, Hentz JG, Magtibay PM, Cornella JL, Magrina JF. Endometriosis: correlation between histologic and visual findings at laparoscopy. *Am J Obstet Gynecol* 2001; **184**:1407–1411; discussion 1411–1403.

Wang K, Li M, Hadley D, Liu R, Glessner J, Grant SF, Hakonarson H, Bucan M. PennCNV: an integrated hidden Markov model designed for high-resolution copy number variation detection in whole-genome SNP genotyping data. *Genome Res* 2007; **17**:1665–1674.

Wang Y, Li Y, Yang Z, Liu K, Wang D. Genome-Wide Microarray Analysis of Long Non-Coding RNAs in Eutopic Secretory Endometrium with Endometriosis. *Cell Physiol Biochem* 2015; **37**:2231–2245.

Wanyonyi SZ, Sequeira E, Mukono SG. Correlation between laparoscopic and histopathologic diagnosis of endometriosis. *Int J Gynaecol Obstet* 2011; **115**:273–276.

Veiga-Castelli LC, Silva JC, Meola J, Ferriani RA, Yoshimoto M, Santos SA, Squire JA, Martelli L. Genomic alterations detected by comparative genomic hybridization in ovarian endometriomas. *Braz J Med Biol Res* 2010; **43**:799–805.

Visscher PM, Brown MA, McCarthy MI, Yang J. Five years of GWAS discovery. *Am J Hum Genet* 2012; **90**:7–24.

Vodolazkaia A, El-Aalamat Y, Popovic D, Mihalyi A, Bossuyt X, Kyama CM, Fassbender A, Bokor A, Schols D, Huskens D, Meuleman C, Peeraer K, Tomassetti C, Gevaert O, Waelkens E, Kasran A, De Moor B, D'Hooghe TM. Evaluation of a panel of 28 biomarkers for the non-invasive diagnosis of endometriosis. *Hum Reprod* 2012; **27**:2698–2711.

Vrba L, Garbe JC, Stampfer MR, Futscher BW. Epigenetic regulation of normal human mammary cell type-specific miRNAs. *Genome Res* 2011; **21**:2026–2037.

Wu Y, Kajdacsy-Balla A, Strawn E, Basir Z, Halverson G, Jailwala P, Wang Y, Wang X, Ghosh S, Guo SW. Transcriptional characterizations of differences between eutopic and ectopic endometrium. *Endocrinology* 2006a; **147**:232–246.

Wu Y, Strawn E, Basir Z, Wang Y, Halverson G, Jailwala P, Guo SW. Genomic alterations in ectopic and eutopic endometria of women with endometriosis. *Gynecol Obstet Invest* 2006b; **62**:148–159.

Yamagata Y, Nishino K, Takaki E, Sato S, Maekawa R, Nakai A, Sugino N. Genome-wide DNA methylation profiling in cultured eutopic and ectopic endometrial stromal cells. *PLoS One* 2014; **9**:e83612.

Yang W, Zhang Y, Fu F, Li R. High-resolution array-comparative genomic hybridization profiling reveals 20q13.33 alterations associated with ovarian endometriosis. *Gynecol Endocrinol* 2013; **29**:603–607.

Young VJ, Brown JK, Saunders PT, Horne AW. The role of the peritoneum in the pathogenesis of endometriosis. *Hum Reprod Update* 2013; **19**:558–569.

ACKNOWLEDGEMENTS

This thesis is a condensation of studies that are conducted with the help and support of many contributors and various organizations that all deserve to be acknowledged. These studies were carried out at the Department of Obstetrics and Gynecology at the University of Tartu and the Competence Centre on Health Technologies, and were supported by the Estonian Ministry of Education and Research, the European Regional Development Fund through the Estonian Centre of Excellence in Genomics, Enterprise Estonia and FP7 Marie Curie Industry-Academia Partnerships and Pathway grant (SARM). Moreover, none of these projects could have been carried out without the help of women willing to participate, the medical personnel of the Tartu University Hospital's Women's Clinic, Nova Vita Clinic and Elite Clinic who helped with patient enrolment and collection of tissue samples. Thank you all!

An African proverb says: "If you want to go fast, go alone. If you want to go far, go together." I have been extremely lucky to have so many good friends and colleagues share this exciting scientific journey with me. Thank you all my friends and colleges, without your help and support I would be still on the road and would have never reached so far! I would like to express my sincerest gratitude to:

- supervisor Professor Andres Salumets for guiding me to the field of reproductive genetics, his unlimited enthusiasm and original ideas.
- supervisor Professor Helle Karro for her valuable professional advice during my doctoral studies.
- supervisor Maire Peters. There are no words that can express my gratitude and appreciation for her guidance and continuous support that kept me well motivated and on the right track. Thank you for your time, your friendship, encouragement and endless patience.
- all my present and former colleagues from the CCHT who have contributed in many ways to the success of these studies. Thank you, Külli, Katrin, Triin, Triin L-P, Kristi, Signe, Anne Mari, Elo, Alar, Mariann, Esta, Jaak, Hardi, Annegrete, Kadri, Marina, Kaarel, Agne and Gerly for your professional advice and good company that made the working environment really enjoyable.
- Triin L-P, who is the kindest person I've ever known. Thank you for your friendship, your sharp mind and willingness to always help with statistics and language corrections.
- Kadri, Elo, Külli and Triin L-P for reviewing this thesis and providing constructive comments.
- co-authors of the studies for their valuable contribution. Especially, I´m really grateful to persons who helped me with statistics and data analyses – Triin Laisk-Podar, Priit Palta, Jaak Simm, Tauno Metsalu, and Vijayachitra Modhukur.
- reviewers Dr. Lili Milani and Dr. Kristiina Rull for their helpful comments.

These acknowledgements would not be complete if I did not thank my parents, family and friends for their precious support. I am really grateful to my parents – Jüri and Rutt Lattik for their unconditional love, support and for always being there for me. Thank you! Also, I would like to express my gratitude to my parents in-law, Enn and Linda, for supporting and taking such a great care of me throughout these years and being wonderful grandparents to my kids. My dear friends Liia, Marju, Helle-Mai and Imbi thank you for fantastic meetings and great discussions. Heidi, thank you for being the best friend and always ready to help and listen. I really enjoy our long walks and great conversations. Last but not least, I truly acknowledge my husband Meelis for his endless patience and support throughout these years. I realize that the life together with a wife who is constantly hurrying, studying, working and travelling and with two little kids is not an easy task. Thank you for everything! Also, I'm very grateful to my nine-year old twins, Merille and Otomar, for spicing up my life in many different ways and for helping me to recognize the real priorities in life.

PUBLICATIONS

CURRICULUM VITAE

Name: Merli Saare
Date of birth: 28.11.1976
Citizenship: Estonian
Telephone: +372 7330402
E-mail: merli.saare@ut.ee

Education:

2011–	University of Tartu, PhD studies in Medical Sciences
2005–2008	University of Tartu, Faculty of Medicine, MSc in biomedicine
2001–2005	University of Tartu, Faculty of Biology and Geography, BSc in molecular diagnostics, gene technology
1993–1995	Hugo Treffner Gymnasium, secondary education

Professional employment:

2010–...	Competence Centre on Health Technologies; researcher
2009–...	University of Tartu, Faculty of Medicine, Institute of Clinical medicine, Department of Obstetrics and Gynecology, researcher
2008–2009	Estonian Biocentre; researcher
2008	University of Tartu, Faculty of Biology and Geography, Institute of Molecular and Cell Biology; researcher

Supervision: Kadri Madissoo, MSc degree in biomedicine, University of Tartu, 2012, (sup) Merli Saare; Andres Salumets; Neeme Tõnisson, "DNA copy number alterations in women with endometriosis".

Publications:

Krjutskov K, Katayama S, Saare M, Vera-Rodriguez M, Lubenets D, Samuel K, Laisk-Podar T, Teder H, Einarsdottir E, Salumets A, Kere J. Single-cell transcriptome analysis of endometrial tissue. *Hum Reprod* 2016.

Saare M, Modhukur V, Suhorutshenko M, Rajashekar B, Rekker K, Soritsa D, Karro H, Soplepmann P, Soritsa A, Lindgren CM, Rahmioglu N, Drong A, Becker CM, Zondervan KT, Salumets A, Peters M. The influence of menstrual cycle and endometriosis on endometrial methylome. *Clin Epigenetics* 2016; **8**:2.

Rekker K, Saare M, Roost AM, Kaart T, Soritsa D, Karro H, Soritsa A, Simon C, Salumets A, Peters M. Circulating miR-200-family micro-RNAs have altered plasma levels in patients with endometriosis and vary with blood collection time. *Fertil Steril* 2015; **104**:938–946 e932.

Soritsa D, Saare M, Laisk-Podar T, Peters M, Soritsa A, Matt K, Karro H, Salumets A. Pregnancy rate in endometriosis patients according to the severity of the disease after using a combined approach of laparoscopy, GnRH agonist treatment and in vitro fertilization. *Gynecol Obstet Invest* 2015; **79**:34–39

Saare M, Rekker K, Laisk-Podar T, Soritsa D, Roost AM, Simm J, Velthut-Meikas A, Samuel K, Metsalu T, Karro H, Soritsa A, Salumets A, Peters M. High-Throughput Sequencing Approach Uncovers the miRNome of Peritoneal Endometriotic Lesions and Adjacent Healthy Tissues. *PLoS One* 2014; **9**:e112630.

Rekker K, Saare M, Roost AM, Kubo AL, Zarovni N, Chiesi A, Salumets A, Peters M. Comparison of serum exosome isolation methods for microRNA profiling. *Clin Biochem* 2014; **47**:135–138.

Rekker K, Saare M, Roost AM, Salumets A, Peters M. Circulating microRNA Profile throughout the menstrual cycle. *PLoS One* 2013; **8**:e81166.

Altmae S, Reimand J, Hovatta O, Zhang P, Kere J, Laisk T, Saare M, Peters M, Vilo J, Stavreus-Evers A, Salumets A. Research resource: interactome of human embryo implantation: identification of gene expression pathways, regulation, and integrated regulatory networks. *Mol Endocrinol* 2012; **26**:203–217.

Lamp M, Saare M, Kadastik U, Karro H, Salumets A, Uibo R, Peters M. Survivin promoter polymorphisms and autoantibodies in endometriosis. *J Reprod Immunol* 2012; **96**:95–100.

Saare M, Soritsa D, Vaidla K, Palta P, Remm M, Laan M, Karro H, Soritsa A, Salumets A, D'Hooghe T, Peters M. No evidence of somatic DNA copy number alterations in eutopic and ectopic endometrial tissue in endometriosis. *Hum Reprod* 2012; **27**:1857–1864.

Saare M, Lamp M, Kadastik Ü, Karro H, Laisk T, Salumets A, Matt K, Peters M. (2011) Endometrioos-anamneesist diagnoosini. Eesti Arst Eesti Arst, 90(7), 321 –326.

Laisk T, Peters M, Saare M, Haller-Kikkatalo K, Karro H, Salumets A. Association of CCR5, TLR2, TLR4 and MBL genetic variations with genital tract infections and tubal factor infertility. *J Reprod Immunol* 2010; **87**:74–81.

Lamp M, Saare M, Laisk T, Karro H, Kadastik U, Metspalu A, Peters M, Salumets A. Genetic variations in vascular endothelial growth factor but not in angiotensin I-converting enzyme genes are associated with endometriosis in Estonian women. *Eur J Obstet Gynecol Reprod Biol* 2010; **153**:85–89.

Peters M, Saare M, Kaart T, Haller-Kikkatalo K, Lend AK, Punab M, Metspalu A, Salumets A. Analysis of polymorphisms in the SRD5A2 gene and semen parameters in Estonian men. *J Androl* 2010; **31**:372–378.

Saare M, Lamp M, Kaart T, Karro H, Kadastik U, Metspalu A, Peters M, Salumets A. Polymorphisms in MMP-2 and MMP-9 promoter regions are associated with endometriosis. *Fertil Steril* 2010; **94**:1560–1563.

Altmae S, Haller K, Peters M, Saare M, Hovatta O, Stavreus-Evers A, Velthut A, Karro H, Metspalu A, Salumets A. Aromatase gene (CYP19A1) variants, female infertility and ovarian stimulation outcome: a preliminary report. *Reprod Biomed Online* 2009; **18**:651–657.

Saare M, Belousova A, Punab M, Peters M, Haller K, Ausmees K, Poolamets O, Karro H, Metspalu A, Salumets A. Androgen receptor gene haplotype is associated with male infertility. *Int J Androl* 2008; **31**:395–402.

Saare M, Lend A.K, Sikut A, Salumets A, Peters M. (2008). Mehe geneetiline viljatus. Eesti Arst, 628–634.

Altmäe S, Haller K, Saare M, Peters M, Karro H, Metspalu A, Salumets A. (2007). Aromataasi CYP19 geeni (TTTA)n variatsioon mõjutab FSH-stimulatsiooni tulemuslikkust lastetuse ravis. Eesti Arst, 86(9), 685–686.

Laasik E, Ojarand M, Pajunen M, Savilahti H, Mae A. Novel mutants of Erwinia carotovora subsp. carotovora defective in the production of plant cell wall degrading enzymes generated by Mu transpososome-mediated insertion mutagenesis. *FEMS Microbiol Lett* 2005; **243**:93–99

ELULOOKIRJELDUS

Nimi: Merli Saare
Sünniaeg: 28.11.1976
Kodakondsus: eestlane
Telefon: +372 7330402
E-mail: merli.saare@ut.ee

Haridustee:

2011–...	Tartu Ülikool doktoriõpingud arstiteadustes
2005–2008	Tartu Ülikool, arstiteaduskond, biomeditsiini magistriõpe
2001–2005	Tartu Ülikool, bioloogia-geograafiateaduskond, geenitehnoloogia eriala
1993–1995	Hugo Treffneri Gümnaasium

Teenistuskäik:

2010–...	Tervisetehnoloogiate Arenduskeskus, teadur
2009–...	Tartu Ülikool, Kliinilise meditsiini instituut, naistekliinik, reproduktiivmeditsiini teadur
2008–2009	Eesti Biokeskus; teadur
2008	Tartu Ülikool, Molekulaar- ja rakubioloogia instituut; teadur

Õppetöö:

Juhendamine: Kadri Madissoo, magistritöö biomeditsiini erialal, Tartu Ülikool, 2012, (juh) Merli Saare; Andres Salumets; Neeme Tõnisson, "DNA koopiaarvu variatsioonid endometrioosiga naistel".

Publikatsioonid:

Krjutskov K, Katayama S, Saare M, Vera-Rodriguez M, Lubenets D, Samuel K, Laisk-Podar T, Teder H, Einarsdottir E, Salumets A, Kere J. Single-cell transcriptome analysis of endometrial tissue. *Hum Reprod* 2016.

Saare M, Modhukur V, Suhorutshenko M, Rajashekar B, Rekker K, Soritsa D, Karro H, Soplepmann P, Soritsa A, Lindgren CM, Rahmioglu N, Drong A, Becker CM, Zondervan KT, Salumets A, Peters M. The influence of menstrual cycle and endometriosis on endometrial methylome. *Clin Epigenetics* 2016; **8**:2.

Rekker K, Saare M, Roost AM, Kaart T, Soritsa D, Karro H, Soritsa A, Simon C, Salumets A, Peters M. Circulating miR-200-family micro-RNAs have altered plasma levels in patients with endometriosis and vary with blood collection time. *Fertil Steril* 2015; **104**:938–946 e932.

Soritsa D, Saare M, Laisk-Podar T, Peters M, Soritsa A, Matt K, Karro H, Salumets A. Pregnancy rate in endometriosis patients according to the severity of the disease after using a combined approach of laparoscopy, GnRH agonist treatment and in vitro fertilization. *Gynecol Obstet Invest* 2015; **79**:34–39

Saare M, Rekker K, Laisk-Podar T, Soritsa D, Roost AM, Simm J, Velthut-Meikas A, Samuel K, Metsalu T, Karro H, Soritsa A, Salumets A, Peters M. High-Throughput Sequencing Approach Uncovers the miRNome of Peritoneal Endometriotic Lesions and Adjacent Healthy Tissues. *PLoS One* 2014; **9**:e112630.

Rekker K, Saare M, Roost AM, Kubo AL, Zarovni N, Chiesi A, Salumets A, Peters M. Comparison of serum exosome isolation methods for microRNA profiling. *Clin Biochem* 2014; **47**:135–138.

Rekker K, Saare M, Roost AM, Salumets A, Peters M. Circulating microRNA Profile throughout the menstrual cycle. *PLoS One* 2013; **8**:e81166.

Altmae S, Reimand J, Hovatta O, Zhang P, Kere J, Laisk T, Saare M, Peters M, Vilo J, Stavreus-Evers A, Salumets A. Research resource: interactome of human embryo implantation: identification of gene expression pathways, regulation, and integrated regulatory networks. *Mol Endocrinol* 2012; **26**:203–217.

Lamp M, Saare M, Kadastik U, Karro H, Salumets A, Uibo R, Peters M. Survivin promoter polymorphisms and autoantibodies in endometriosis. *J Reprod Immunol* 2012; **96**:95–100.

Saare M, Soritsa D, Vaidla K, Palta P, Remm M, Laan M, Karro H, Soritsa A, Salumets A, D'Hooghe T, Peters M. No evidence of somatic DNA copy number alterations in eutopic and ectopic endometrial tissue in endometriosis. *Hum Reprod* 2012; **27**:1857–1864.

Saare M, Lamp M, Kadastik Ü, Karro H, Laisk T, Salumets A, Matt K, Peters M. (2011) Endometrioos-anamneesist diagnoosini. Eesti Arst Eesti Arst, 90(7), 321–326.

Laisk T, Peters M, Saare M, Haller-Kikkatalo K, Karro H, Salumets A. Association of CCR5, TLR2, TLR4 and MBL genetic variations with genital tract infections and tubal factor infertility. *J Reprod Immunol* 2010; **87**:74–81.

Lamp M, Saare M, Laisk T, Karro H, Kadastik U, Metspalu A, Peters M, Salumets A. Genetic variations in vascular endothelial growth factor but not in angiotensin I-converting enzyme genes are associated with endometriosis in Estonian women. *Eur J Obstet Gynecol Reprod Biol* 2010; **153**:85–89.

Peters M, Saare M, Kaart T, Haller-Kikkatalo K, Lend AK, Punab M, Metspalu A, Salumets A. Analysis of polymorphisms in the SRD5A2 gene and semen parameters in Estonian men. *J Androl* 2010; **31**:372–378.

Saare M, Lamp M, Kaart T, Karro H, Kadastik U, Metspalu A, Peters M, Salumets A. Polymorphisms in MMP-2 and MMP-9 promoter regions are associated with endometriosis. *Fertil Steril* 2010; **94**:1560–1563.

Altmae S, Haller K, Peters M, Saare M, Hovatta O, Stavreus-Evers A, Velthut A, Karro H, Metspalu A, Salumets A. Aromatase gene (CYP19A1) variants, female infertility and ovarian stimulation outcome: a preliminary report. *Reprod Biomed Online* 2009; **18**:651–657.

Saare M, Belousova A, Punab M, Peters M, Haller K, Ausmees K, Poolamets O, Karro H, Metspalu A, Salumets A. Androgen receptor gene haplotype is associated with male infertility. *Int J Androl* 2008; **31**:395–402.

Saare M, Lend A.K, Sikut A, Salumets A, Peters M. (2008). Mehe geneetiline viljatus. Eesti Arst, 628–634.

Altmäe S, Haller K, Saare M, Peters M, Karro H, Metspalu A, Salumets A. (2007). Aromataasi CYP19 geeni (TTTA)n variatsioon mõjutab FSH-stimulatsiooni tulemuslikkust lastetuse ravis. Eesti Arst, 86(9), 685–686.

Laasik E, Ojarand M, Pajunen M, Savilahti H, Mae A. Novel mutants of Erwinia carotovora subsp. carotovora defective in the production of plant cell wall degrading enzymes generated by Mu transpososome-mediated insertion mutagenesis. *FEMS Microbiol Lett* 2005; **243**:93–99

DISSERTATIONES MEDICINAE UNIVERSITATIS TARTUENSIS

1. **Heidi-Ingrid Maaroos**. The natural course of gastric ulcer in connection with chronic gastritis and *Helicobacter pylori*. Tartu, 1991.
2. **Mihkel Zilmer**. Na-pump in normal and tumorous brain tissues: Structural, functional and tumorigenesis aspects. Tartu, 1991.
3. **Eero Vasar**. Role of cholecystokinin receptors in the regulation of behaviour and in the action of haloperidol and diazepam. Tartu, 1992.
4. **Tiina Talvik**. Hypoxic-ischaemic brain damage in neonates (clinical, biochemical and brain computed tomographical investigation). Tartu, 1992.
5. **Ants Peetsalu**. Vagotomy in duodenal ulcer disease: A study of gastric acidity, serum pepsinogen I, gastric mucosal histology and *Helicobacter pylori*. Tartu, 1992.
6. **Marika Mikelsaar**. Evaluation of the gastrointestinal microbial ecosystem in health and disease. Tartu, 1992.
7. **Hele Everaus**. Immuno-hormonal interactions in chronic lymphocytic leukaemia and multiple myeloma. Tartu, 1993.
8. **Ruth Mikelsaar**. Etiological factors of diseases in genetically consulted children and newborn screening: dissertation for the commencement of the degree of doctor of medical sciences. Tartu, 1993.
9. **Agu Tamm**. On metabolic action of intestinal microflora: clinical aspects. Tartu, 1993.
10. **Katrin Gross**. Multiple sclerosis in South-Estonia (epidemiological and computed tomographical investigations). Tartu, 1993.
11. **Oivi Uibo**. Childhood coeliac disease in Estonia: occurrence, screening, diagnosis and clinical characterization. Tartu, 1994.
12. **Viiu Tuulik**. The functional disorders of central nervous system of chemistry workers. Tartu, 1994.
13. **Margus Viigimaa**. Primary haemostasis, antiaggregative and anticoagulant treatment of acute myocardial infarction. Tartu, 1994.
14. **Rein Kolk**. Atrial versus ventricular pacing in patients with sick sinus syndrome. Tartu, 1994.
15. **Toomas Podar**. Incidence of childhood onset type 1 diabetes mellitus in Estonia. Tartu, 1994.
16. **Kiira Subi**. The laboratory surveillance of the acute respiratory viral infections in Estonia. Tartu, 1995.
17. **Irja Lutsar**. Infections of the central nervous system in children (epidemiologic, diagnostic and therapeutic aspects, long term outcome). Tartu, 1995.
18. **Aavo Lang**. The role of dopamine, 5-hydroxytryptamine, sigma and NMDA receptors in the action of antipsychotic drugs. Tartu, 1995.
19. **Andrus Arak**. Factors influencing the survival of patients after radical surgery for gastric cancer. Tartu, 1996.

20. **Tõnis Karki**. Quantitative composition of the human lactoflora and method for its examination. Tartu, 1996.
21. **Reet Mändar**. Vaginal microflora during pregnancy and its transmission to newborn. Tartu, 1996.
22. **Triin Remmel**. Primary biliary cirrhosis in Estonia: epidemiology, clinical characterization and prognostication of the course of the disease. Tartu, 1996.
23. **Toomas Kivastik**. Mechanisms of drug addiction: focus on positive reinforcing properties of morphine. Tartu, 1996.
24. **Paavo Pokk**. Stress due to sleep deprivation: focus on $GABA_A$ receptor-chloride ionophore complex. Tartu, 1996.
25. **Kristina Allikmets**. Renin system activity in essential hypertension. Associations with atherothrombogenic cardiovascular risk factors and with the efficacy of calcium antagonist treatment. Tartu, 1996.
26. **Triin Parik**. Oxidative stress in essential hypertension: Associations with metabolic disturbances and the effects of calcium antagonist treatment. Tartu, 1996.
27. **Svetlana Päi**. Factors promoting heterogeneity of the course of rheumatoid arthritis. Tartu, 1997.
28. **Maarike Sallo**. Studies on habitual physical activity and aerobic fitness in 4 to 10 years old children. Tartu, 1997.
29. **Paul Naaber**. *Clostridium difficile* infection and intestinal microbial ecology. Tartu, 1997.
30. **Rein Pähkla**. Studies in pinoline pharmacology. Tartu, 1997.
31. **Andrus Juhan Voitk**. Outpatient laparoscopic cholecystectomy. Tartu, 1997.
32. **Joel Starkopf**. Oxidative stress and ischaemia-reperfusion of the heart. Tartu, 1997.
33. **Janika Kõrv**. Incidence, case-fatality and outcome of stroke. Tartu, 1998.
34. **Ülla Linnamägi**. Changes in local cerebral blood flow and lipid peroxidation following lead exposure in experiment. Tartu, 1998.
35. **Ave Minajeva**. Sarcoplasmic reticulum function: comparison of atrial and ventricular myocardium. Tartu, 1998.
36. **Oleg Milenin**. Reconstruction of cervical part of esophagus by revascularised ileal autografts in dogs. A new complex multistage method. Tartu, 1998.
37. **Sergei Pakriev**. Prevalence of depression, harmful use of alcohol and alcohol dependence among rural population in Udmurtia. Tartu, 1998.
38. **Allen Kaasik**. Thyroid hormone control over β-adrenergic signalling system in rat atria. Tartu, 1998.
39. **Vallo Matto**. Pharmacological studies on anxiogenic and antiaggressive properties of antidepressants. Tartu, 1998.
40. **Maire Vasar**. Allergic diseases and bronchial hyperreactivity in Estonian children in relation to environmental influences. Tartu, 1998.
41. **Kaja Julge**. Humoral immune responses to allergens in early childhood. Tartu, 1998.

42. **Heli Grünberg**. The cardiovascular risk of Estonian schoolchildren. A cross-sectional study of 9-, 12- and 15-year-old children. Tartu, 1998.
43. **Epp Sepp**. Formation of intestinal microbial ecosystem in children. Tartu, 1998.
44. **Mai Ots**. Characteristics of the progression of human and experimental glomerulopathies. Tartu, 1998.
45. **Tiina Ristimäe**. Heart rate variability in patients with coronary artery disease. Tartu, 1998.
46. **Leho Kõiv**. Reaction of the sympatho-adrenal and hypothalamo-pituitary-adrenocortical system in the acute stage of head injury. Tartu, 1998.
47. **Bela Adojaan**. Immune and genetic factors of childhood onset IDDM in Estonia. An epidemiological study. Tartu, 1999.
48. **Jakov Shlik**. Psychophysiological effects of cholecystokinin in humans. Tartu, 1999.
49. **Kai Kisand**. Autoantibodies against dehydrogenases of α-ketoacids. Tartu, 1999.
50. **Toomas Marandi**. Drug treatment of depression in Estonia. Tartu, 1999.
51. **Ants Kask**. Behavioural studies on neuropeptide Y. Tartu, 1999.
52. **Ello-Rahel Karelson**. Modulation of adenylate cyclase activity in the rat hippocampus by neuropeptide galanin and its chimeric analogs. Tartu, 1999.
53. **Tanel Laisaar**. Treatment of pleural empyema — special reference to intrapleural therapy with streptokinase and surgical treatment modalities. Tartu, 1999.
54. **Eve Pihl**. Cardiovascular risk factors in middle-aged former athletes. Tartu, 1999.
55. **Katrin Õunap**. Phenylketonuria in Estonia: incidence, newborn screening, diagnosis, clinical characterization and genotype/phenotype correlation. Tartu, 1999.
56. **Siiri Kõljalg**. *Acinetobacter* – an important nosocomial pathogen. Tartu, 1999.
57. **Helle Karro**. Reproductive health and pregnancy outcome in Estonia: association with different factors. Tartu, 1999.
58. **Heili Varendi**. Behavioral effects observed in human newborns during exposure to naturally occurring odors. Tartu, 1999.
59. **Anneli Beilmann**. Epidemiology of epilepsy in children and adolescents in Estonia. Prevalence, incidence, and clinical characteristics. Tartu, 1999.
60. **Vallo Volke**. Pharmacological and biochemical studies on nitric oxide in the regulation of behaviour. Tartu, 1999.
61. **Pilvi Ilves**. Hypoxic-ischaemic encephalopathy in asphyxiated term infants. A prospective clinical, biochemical, ultrasonographical study. Tartu, 1999.
62. **Anti Kalda**. Oxygen-glucose deprivation-induced neuronal death and its pharmacological prevention in cerebellar granule cells. Tartu, 1999.
63. **Eve-Irene Lepist**. Oral peptide prodrugs – studies on stability and absorption. Tartu, 2000.

64. **Jana Kivastik**. Lung function in Estonian schoolchildren: relationship with anthropometric indices and respiratory symptomas, reference values for dynamic spirometry. Tartu, 2000.
65. **Karin Kull**. Inflammatory bowel disease: an immunogenetic study. Tartu, 2000.
66. **Kaire Innos**. Epidemiological resources in Estonia: data sources, their quality and feasibility of cohort studies. Tartu, 2000.
67. **Tamara Vorobjova**. Immune response to *Helicobacter pylori* and its association with dynamics of chronic gastritis and epithelial cell turnover in antrum and corpus. Tartu, 2001.
68. **Ruth Kalda**. Structure and outcome of family practice quality in the changing health care system of Estonia. Tartu, 2001.
69. **Annika Krüüner**. *Mycobacterium tuberculosis* – spread and drug resistance in Estonia. Tartu, 2001.
70. **Marlit Veldi**. Obstructive Sleep Apnoea: Computerized Endopharyngeal Myotonometry of the Soft Palate and Lingual Musculature. Tartu, 2001.
71. **Anneli Uusküla**. Epidemiology of sexually transmitted diseases in Estonia in 1990–2000. Tartu, 2001.
72. **Ade Kallas**. Characterization of antibodies to coagulation factor VIII. Tartu, 2002.
73. **Heidi Annuk**. Selection of medicinal plants and intestinal lactobacilli as antimicrobil components for functional foods. Tartu, 2002.
74. **Aet Lukmann**. Early rehabilitation of patients with ischaemic heart disease after surgical revascularization of the myocardium: assessment of health-related quality of life, cardiopulmonary reserve and oxidative stress. A clinical study. Tartu, 2002.
75. **Maigi Eisen**. Pathogenesis of Contact Dermatitis: participation of Oxidative Stress. A clinical – biochemical study. Tartu, 2002.
76. **Piret Hussar**. Histology of the post-traumatic bone repair in rats. Elaboration and use of a new standardized experimental model – bicortical perforation of tibia compared to internal fracture and resection osteotomy. Tartu, 2002.
77. **Tõnu Rätsep**. Aneurysmal subarachnoid haemorrhage: Noninvasive monitoring of cerebral haemodynamics. Tartu, 2002.
78. **Marju Herodes**. Quality of life of people with epilepsy in Estonia. Tartu, 2003.
79. **Katre Maasalu**. Changes in bone quality due to age and genetic disorders and their clinical expressions in Estonia. Tartu, 2003.
80. **Toomas Sillakivi**. Perforated peptic ulcer in Estonia: epidemiology, risk factors and relations with *Helicobacter pylori*. Tartu, 2003.
81. **Leena Puksa**. Late responses in motor nerve conduction studies. F and A waves in normal subjects and patients with neuropathies. Tartu, 2003.
82. **Krista Lõivukene**. *Helicobacter pylori* in gastric microbial ecology and its antimicrobial susceptibility pattern. Tartu, 2003.

83. **Helgi Kolk**. Dyspepsia and *Helicobacter pylori* infection: the diagnostic value of symptoms, treatment and follow-up of patients referred for upper gastrointestinal endoscopy by family physicians. Tartu, 2003.
84. **Helena Soomer**. Validation of identification and age estimation methods in forensic odontology. Tartu, 2003.
85. **Kersti Oselin**. Studies on the human MDR1, MRP1, and MRP2 ABC transporters: functional relevance of the genetic polymorphisms in the *MDR1* and *MRP1* gene. Tartu, 2003.
86. **Jaan Soplepmann**. Peptic ulcer haemorrhage in Estonia: epidemiology, prognostic factors, treatment and outcome. Tartu, 2003.
87. **Margot Peetsalu**. Long-term follow-up after vagotomy in duodenal ulcer disease: recurrent ulcer, changes in the function, morphology and *Helicobacter pylori* colonisation of the gastric mucosa. Tartu, 2003.
88. **Kersti Klaamas**. Humoral immune response to *Helicobacter pylori* a study of host-dependent and microbial factors. Tartu, 2003.
89. **Pille Taba**. Epidemiology of Parkinson's disease in Tartu, Estonia. Prevalence, incidence, clinical characteristics, and pharmacoepidemiology. Tartu, 2003.
90. **Alar Veraksitš**. Characterization of behavioural and biochemical phenotype of cholecystokinin-2 receptor deficient mice: changes in the function of the dopamine and endopioidergic system. Tartu, 2003.
91. **Ingrid Kalev**. CC-chemokine receptor 5 (CCR5) gene polymorphism in Estonians and in patients with Type I and Type II diabetes mellitus. Tartu, 2003.
92. **Lumme Kadaja**. Molecular approach to the regulation of mitochondrial function in oxidative muscle cells. Tartu, 2003.
93. **Aive Liigant**. Epidemiology of primary central nervous system tumours in Estonia from 1986 to 1996. Clinical characteristics, incidence, survival and prognostic factors. Tartu, 2004.
94. **Andres, Kulla**. Molecular characteristics of mesenchymal stroma in human astrocytic gliomas. Tartu, 2004.
95. **Mari Järvelaid**. Health damaging risk behaviours in adolescence. Tartu, 2004.
96. **Ülle Pechter**. Progression prevention strategies in chronic renal failure and hypertension. An experimental and clinical study. Tartu, 2004.
97. **Gunnar Tasa**. Polymorphic glutathione S-transferases – biology and role in modifying genetic susceptibility to senile cataract and primary open angle glaucoma. Tartu, 2004.
98. **Tuuli Käämbre**. Intracellular energetic unit: structural and functional aspects. Tartu, 2004.
99. **Vitali Vassiljev**. Influence of nitric oxide syntase inhibitors on the effects of ethanol after acute and chronic ethanol administration and withdrawal. Tartu, 2004.

100. **Aune Rehema**. Assessment of nonhaem ferrous iron and glutathione redox ratio as markers of pathogeneticity of oxidative stress in different clinical groups. Tartu, 2004.
101. **Evelin Seppet**. Interaction of mitochondria and ATPases in oxidative muscle cells in normal and pathological conditions. Tartu, 2004.
102. **Eduard Maron**. Serotonin function in panic disorder: from clinical experiments to brain imaging and genetics. Tartu, 2004.
103. **Marje Oona**. *Helicobacter pylori* infection in children: epidemiological and therapeutic aspects. Tartu, 2004.
104. **Kersti Kokk**. Regulation of active and passive molecular transport in the testis. Tartu, 2005.
105. **Vladimir Järv**. Cross-sectional imaging for pretreatment evaluation and follow-up of pelvic malignant tumours. Tartu, 2005.
106. **Andre Õun**. Epidemiology of adult epilepsy in Tartu, Estonia. Incidence, prevalence and medical treatment. Tartu, 2005.
107. **Piibe Muda**. Homocysteine and hypertension: associations between homocysteine and essential hypertension in treated and untreated hypertensive patients with and without coronary artery disease. Tartu, 2005.
108. **Külli Kingo**. The interleukin-10 family cytokines gene polymorphisms in plaque psoriasis. Tartu, 2005.
109. **Mati Merila**. Anatomy and clinical relevance of the glenohumeral joint capsule and ligaments. Tartu, 2005.
110. **Epp Songisepp**. Evaluation of technological and functional properties of the new probiotic *Lactobacillus fermentum* ME-3. Tartu, 2005.
111. **Tiia Ainla**. Acute myocardial infarction in Estonia: clinical characteristics, management and outcome. Tartu, 2005.
112. **Andres Sell**. Determining the minimum local anaesthetic requirements for hip replacement surgery under spinal anaesthesia – a study employing a spinal catheter. Tartu, 2005.
113. **Tiia Tamme**. Epidemiology of odontogenic tumours in Estonia. Pathogenesis and clinical behaviour of ameloblastoma. Tartu, 2005.
114. **Triine Annus**. Allergy in Estonian schoolchildren: time trends and characteristics. Tartu, 2005.
115. **Tiia Voor**. Microorganisms in infancy and development of allergy: comparison of Estonian and Swedish children. Tartu, 2005.
116. **Priit Kasenõmm**. Indicators for tonsillectomy in adults with recurrent tonsillitis – clinical, microbiological and pathomorphological investigations. Tartu, 2005.
117. **Eva Zusinaite**. Hepatitis C virus: genotype identification and interactions between viral proteases. Tartu, 2005.
118. **Piret Kõll**. Oral lactoflora in chronic periodontitis and periodontal health. Tartu, 2006.
119. **Tiina Stelmach**. Epidemiology of cerebral palsy and unfavourable neurodevelopmental outcome in child population of Tartu city and county, Estonia Prevalence, clinical features and risk factors. Tartu, 2006.

120. **Katrin Pudersell**. Tropane alkaloid production and riboflavine excretion in the field and tissue cultures of henbane (*Hyoscyamus niger* L.). Tartu, 2006.
121. **Külli Jaako**. Studies on the role of neurogenesis in brain plasticity. Tartu, 2006.
122. **Aare Märtson**. Lower limb lengthening: experimental studies of bone regeneration and long-term clinical results. Tartu, 2006.
123. **Heli Tähepõld**. Patient consultation in family medicine. Tartu, 2006.
124. **Stanislav Liskmann**. Peri-implant disease: pathogenesis, diagnosis and treatment in view of both inflammation and oxidative stress profiling. Tartu, 2006.
125. **Ruth Rudissaar**. Neuropharmacology of atypical antipsychotics and an animal model of psychosis. Tartu, 2006.
126. **Helena Andreson**. Diversity of *Helicobacter pylori* genotypes in Estonian patients with chronic inflammatory gastric diseases. Tartu, 2006.
127. **Katrin Pruus**. Mechanism of action of antidepressants: aspects of serotoninergic system and its interaction with glutamate. Tartu, 2006.
128. **Priit Põder**. Clinical and experimental investigation: relationship of ischaemia/reperfusion injury with oxidative stress in abdominal aortic aneurysm repair and in extracranial brain artery endarterectomy and possibilities of protection against ischaemia using a glutathione analogue in a rat model of global brain ischaemia. Tartu, 2006.
129. **Marika Tammaru**. Patient-reported outcome measurement in rheumatoid arthritis. Tartu, 2006.
130. **Tiia Reimand**. Down syndrome in Estonia. Tartu, 2006.
131. **Diva Eensoo**. Risk-taking in traffic and Markers of Risk-Taking Behaviour in Schoolchildren and Car Drivers. Tartu, 2007.
132. **Riina Vibo**. The third stroke registry in Tartu, Estonia from 2001 to 2003: incidence, case-fatality, risk factors and long-term outcome. Tartu, 2007.
133. **Chris Pruunsild**. Juvenile idiopathic arthritis in children in Estonia. Tartu, 2007.
134. **Eve Õiglane-Šlik**. Angelman and Prader-Willi syndromes in Estonia. Tartu, 2007.
135. **Kadri Haller**. Antibodies to follicle stimulating hormone. Significance in female infertility. Tartu, 2007.
136. **Pille Ööpik**. Management of depression in family medicine. Tartu, 2007.
137. **Jaak Kals**. Endothelial function and arterial stiffness in patients with atherosclerosis and in healthy subjects. Tartu, 2007.
138. **Priit Kampus**. Impact of inflammation, oxidative stress and age on arterial stiffness and carotid artery intima-media thickness. Tartu, 2007.
139. **Margus Punab**. Male fertility and its risk factors in Estonia. Tartu, 2007.
140. **Alar Toom**. Heterotopic ossification after total hip arthroplasty: clinical and pathogenetic investigation. Tartu, 2007.

141. **Lea Pehme**. Epidemiology of tuberculosis in Estonia 1991–2003 with special regard to extrapulmonary tuberculosis and delay in diagnosis of pulmonary tuberculosis. Tartu, 2007.
142. **Juri Karjagin**. The pharmacokinetics of metronidazole and meropenem in septic shock. Tartu, 2007.
143. **Inga Talvik**. Inflicted traumatic brain injury shaken baby syndrome in Estonia – epidemiology and outcome. Tartu, 2007.
144. **Tarvo Rajasalu**. Autoimmune diabetes: an immunological study of type 1 diabetes in humans and in a model of experimental diabetes (in RIP-B7.1 mice). Tartu, 2007.
145. **Inga Karu**. Ischaemia-reperfusion injury of the heart during coronary surgery: a clinical study investigating the effect of hyperoxia. Tartu, 2007.
146. **Peeter Padrik**. Renal cell carcinoma: Changes in natural history and treatment of metastatic disease. Tartu, 2007.
147. **Neve Vendt**. Iron deficiency and iron deficiency anaemia in infants aged 9 to 12 months in Estonia. Tartu, 2008.
148. **Lenne-Triin Heidmets**. The effects of neurotoxins on brain plasticity: focus on neural Cell Adhesion Molecule. Tartu, 2008.
149. **Paul Korrovits**. Asymptomatic inflammatory prostatitis: prevalence, etiological factors, diagnostic tools. Tartu, 2008.
150. **Annika Reintam**. Gastrointestinal failure in intensive care patients. Tartu, 2008.
151. **Kristiina Roots**. Cationic regulation of Na-pump in the normal, Alzheimer's and CCK_2 receptor-deficient brain. Tartu, 2008.
152. **Helen Puusepp**. The genetic causes of mental retardation in Estonia: fragile X syndrome and creatine transporter defect. Tartu, 2009.
153. **Kristiina Rull**. Human chorionic gonadotropin beta genes and recurrent miscarriage: expression and variation study. Tartu, 2009.
154. **Margus Eimre**. Organization of energy transfer and feedback regulation in oxidative muscle cells. Tartu, 2009.
155. **Maire Link**. Transcription factors FoxP3 and AIRE: autoantibody associations. Tartu, 2009.
156. **Kai Haldre**. Sexual health and behaviour of young women in Estonia. Tartu, 2009.
157. **Kaur Liivak**. Classical form of congenital adrenal hyperplasia due to 21-hydroxylase deficiency in Estonia: incidence, genotype and phenotype with special attention to short-term growth and 24-hour blood pressure. Tartu, 2009.
158. **Kersti Ehrlich**. Antioxidative glutathione analogues (UPF peptides) – molecular design, structure-activity relationships and testing the protective properties. Tartu, 2009.
159. **Anneli Rätsep**. Type 2 diabetes care in family medicine. Tartu, 2009.
160. **Silver Türk**. Etiopathogenetic aspects of chronic prostatitis: role of mycoplasmas, coryneform bacteria and oxidative stress. Tartu, 2009.

161. **Kaire Heilman**. Risk markers for cardiovascular disease and low bone mineral density in children with type 1 diabetes. Tartu, 2009.
162. **Kristi Rüütel**. HIV-epidemic in Estonia: injecting drug use and quality of life of people living with HIV. Tartu, 2009.
163. **Triin Eller**. Immune markers in major depression and in antidepressive treatment. Tartu, 2009.
164. **Siim Suutre**. The role of TGF-β isoforms and osteoprogenitor cells in the pathogenesis of heterotopic ossification. An experimental and clinical study of hip arthroplasty. Tartu, 2010.
165. **Kai Kliiman**. Highly drug-resistant tuberculosis in Estonia: Risk factors and predictors of poor treatment outcome. Tartu, 2010.
166. **Inga Villa**. Cardiovascular health-related nutrition, physical activity and fitness in Estonia. Tartu, 2010.
167. **Tõnis Org**. Molecular function of the first PHD finger domain of Autoimmune Regulator protein. Tartu, 2010.
168. **Tuuli Metsvaht**. Optimal antibacterial therapy of neonates at risk of early onset sepsis. Tartu, 2010.
169. **Jaanus Kahu**. Kidney transplantation: Studies on donor risk factors and mycophenolate mofetil. Tartu, 2010.
170. **Koit Reimand**. Autoimmunity in reproductive failure: A study on associated autoantibodies and autoantigens. Tartu, 2010.
171. **Mart Kull**. Impact of vitamin D and hypolactasia on bone mineral density: a population based study in Estonia. Tartu, 2010.
172. **Rael Laugesaar**. Stroke in children – epidemiology and risk factors. Tartu, 2010.
173. **Mark Braschinsky**. Epidemiology and quality of life issues of hereditary spastic paraplegia in Estonia and implemention of genetic analysis in everyday neurologic practice. Tartu, 2010.
174. **Kadri Suija**. Major depression in family medicine: associated factors, recurrence and possible intervention. Tartu, 2010.
175. **Jarno Habicht**. Health care utilisation in Estonia: socioeconomic determinants and financial burden of out-of-pocket payments. Tartu, 2010.
176. **Kristi Abram**. The prevalence and risk factors of rosacea. Subjective disease perception of rosacea patients. Tartu, 2010.
177. **Malle Kuum**. Mitochondrial and endoplasmic reticulum cation fluxes: Novel roles in cellular physiology. Tartu, 2010.
178. **Rita Teek**. The genetic causes of early onset hearing loss in Estonian children. Tartu, 2010.
179. **Daisy Volmer**. The development of community pharmacy services in Estonia – public and professional perceptions 1993–2006. Tartu, 2010.
180. **Jelena Lissitsina**. Cytogenetic causes in male infertility. Tartu, 2011.
181. **Delia Lepik**. Comparison of gunshot injuries caused from Tokarev, Makarov and Glock 19 pistols at different firing distances. Tartu, 2011.
182. **Ene-Renate Pähkla**. Factors related to the efficiency of treatment of advanced periodontitis. Tartu, 2011.

183. **Maarja Krass**. L-Arginine pathways and antidepressant action. Tartu, 2011.
184. **Taavi Lai**. Population health measures to support evidence-based health policy in Estonia. Tartu, 2011.
185. **Tiit Salum**. Similarity and difference of temperature-dependence of the brain sodium pump in normal, different neuropathological, and aberrant conditions and its possible reasons. Tartu, 2011.
186. **Tõnu Vooder**. Molecular differences and similarities between histological subtypes of non-small cell lung cancer. Tartu, 2011.
187. **Jelena Štšepetova**. The characterisation of intestinal lactic acid bacteria using bacteriological, biochemical and molecular approaches. Tartu, 2011.
188. **Radko Avi**. Natural polymorphisms and transmitted drug resistance in Estonian HIV-1 CRF06_cpx and its recombinant viruses. Tartu, 2011, 116 p.
189. **Edward Laane**. Multiparameter flow cytometry in haematological malignancies. Tartu, 2011, 152 p.
190. **Triin Jagomägi**. A study of the genetic etiology of nonsyndromic cleft lip and palate. Tartu, 2011, 158 p.
191. **Ivo Laidmäe**. Fibrin glue of fish (*Salmo salar*) origin: immunological study and development of new pharmaceutical preparation. Tartu, 2012, 150 p.
192. **Ülle Parm**. Early mucosal colonisation and its role in prediction of invasive infection in neonates at risk of early onset sepsis. Tartu, 2012, 168 p.
193. **Kaupo Teesalu**. Autoantibodies against desmin and transglutaminase 2 in celiac disease: diagnostic and functional significance. Tartu, 2012, 142 p.
194. **Maksim Zagura**. Biochemical, functional and structural profiling of arterial damage in atherosclerosis. Tartu, 2012, 162 p.
195. **Vivian Kont**. Autoimmune regulator: characterization of thymic gene regulation and promoter methylation. Tartu, 2012, 134 p.
196. **Pirje Hütt**. Functional properties, persistence, safety and efficacy of potential probiotic lactobacilli. Tartu, 2012, 246 p.
197. **Innar Tõru**. Serotonergic modulation of CCK-4- induced panic. Tartu, 2012, 132 p.
198. **Sigrid Vorobjov**. Drug use, related risk behaviour and harm reduction interventions utilization among injecting drug users in Estonia: implications for drug policy. Tartu, 2012, 120 p.
199. **Martin Serg**. Therapeutic aspects of central haemodynamics, arterial stiffness and oxidative stress in hypertension. Tartu, 2012, 156 p.
200. **Jaanika Kumm**. Molecular markers of articular tissues in early knee osteoarthritis: a population-based longitudinal study in middle-aged subjects. Tartu, 2012, 159 p.
201. **Kertu Rünkorg**. Functional changes of dopamine, endopioid and endocannabinoid systems in CCK2 receptor deficient mice. Tartu, 2012, 125 p.
202. **Mai Blöndal**. Changes in the baseline characteristics, management and outcomes of acute myocardial infarction in Estonia. Tartu, 2012, 127 p.

203. **Jana Lass**. Epidemiological and clinical aspects of medicines use in children in Estonia. Tartu, 2012, 170 p.
204. **Kai Truusalu**. Probiotic lactobacilli in experimental persistent *Salmonella* infection. Tartu, 2013, 139 p.
205. **Oksana Jagur**. Temporomandibular joint diagnostic imaging in relation to pain and bone characteristics. Long-term results of arthroscopic treatment. Tartu, 2013, 126 p.
206. **Katrin Sikk**. Manganese-ephedrone intoxication – pathogenesis of neurological damage and clinical symptomatology. Tartu, 2013, 125 p.
207. **Kai Blöndal**. Tuberculosis in Estonia with special emphasis on drug-resistant tuberculosis: Notification rate, disease recurrence and mortality. Tartu, 2013, 151 p.
208. **Marju Puurand**. Oxidative phosphorylation in different diseases of gastric mucosa. Tartu, 2013, 123 p.
209. **Aili Tagoma**. Immune activation in female infertility: Significance of autoantibodies and inflammatory mediators. Tartu, 2013, 135 p.
210. **Liis Sabre**. Epidemiology of traumatic spinal cord injury in Estonia. Brain activation in the acute phase of traumatic spinal cord injury. Tartu, 2013, 135 p.
211. **Merit Lamp**. Genetic susceptibility factors in endometriosis. Tartu, 2013, 125 p.
212. **Erik Salum**. Beneficial effects of vitamin D and angiotensin II receptor blocker on arterial damage. Tartu, 2013, 167 p.
213. **Maire Karelson**. Vitiligo: clinical aspects, quality of life and the role of melanocortin system in pathogenesis. Tartu, 2013, 153 p.
214. **Kuldar Kaljurand**. Prevalence of exfoliation syndrome in Estonia and its clinical significance. Tartu, 2013, 113 p.
215. **Raido Paasma**. Clinical study of methanol poisoning: handling large outbreaks, treatment with antidotes, and long-term outcomes. Tartu, 2013, 96 p.
216. **Anne Kleinberg**. Major depression in Estonia: prevalence, associated factors, and use of health services. Tartu, 2013, 129 p.
217. **Triin Eglit**. Obesity, impaired glucose regulation, metabolic syndrome and their associations with high-molecular-weight adiponectin levels. Tartu, 2014, 115 p.
218. **Kristo Ausmees**. Reproductive function in middle-aged males: Associations with prostate, lifestyle and couple infertility status. Tartu, 2014, 125 p.
219. **Kristi Huik**. The influence of host genetic factors on the susceptibility to HIV and HCV infections among intravenous drug users. Tartu, 2014, 144 p.
220. **Liina Tserel**. Epigenetic profiles of monocytes, monocyte-derived macrophages and dendritic cells. Tartu, 2014, 143 p.
221. **Irina Kerna**. The contribution of *ADAM12* and *CILP* genes to the development of knee osteoarthritis. Tartu, 2014, 152 p.

222. **Ingrit Liiv**. Autoimmune regulator protein interaction with DNA-dependent protein kinase and its role in apoptosis. Tartu, 2014, 143 p.
223. **Liivi Maddison**. Tissue perfusion and metabolism during intra-abdominal hypertension. Tartu, 2014, 103 p.
224. **Krista Ress**. Childhood coeliac disease in Estonia, prevalence in atopic dermatitis and immunological characterisation of coexistence. Tartu, 2014, 124 p.
225. **Kai Muru**. Prenatal screening strategies, long-term outcome of children with marked changes in maternal screening tests and the most common syndromic heart anomalies in Estonia. Tartu, 2014, 189 p.
226. **Kaja Rahu**. Morbidity and mortality among Baltic Chernobyl cleanup workers: a register-based cohort study. Tartu, 2014, 155 p.
227. **Klari Noormets**. The development of diabetes mellitus, fertility and energy metabolism disturbances in a Wfs1-deficient mouse model of Wolfram syndrome. Tartu, 2014, 132 p.
228. **Liis Toome**. Very low gestational age infants in Estonia. Tartu, 2014, 183 p.
229. **Ceith Nikkolo**. Impact of different mesh parameters on chronic pain and foreign body feeling after open inguinal hernia repair. Tartu, 2014, 132 p.
230. **Vadim Brjalin**. Chronic hepatitis C: predictors of treatment response in Estonian patients. Tartu, 2014, 122 p.
231. **Vahur Metsna**. Anterior knee pain in patients following total knee arthroplasty: the prevalence, correlation with patellar cartilage impairment and aspects of patellofemoral congruence. Tartu, 2014, 130 p.
232. **Marju Kase**. Glioblastoma multiforme: possibilities to improve treatment efficacy. Tartu, 2015, 137 p.
233. **Riina Runnel**. Oral health among elementary school children and the effects of polyol candies on the prevention of dental caries. Tartu, 2015, 112 p.
234. **Made Laanpere**. Factors influencing women's sexual health and reproductive choices in Estonia. Tartu, 2015, 176 p.
235. **Andres Lust**. Water mediated solid state transformations of a polymorphic drug – effect on pharmaceutical product performance. Tartu, 2015, 134 p.
236. **Anna Klugman**. Functionality related characterization of pretreated wood lignin, cellulose and polyvinylpyrrolidone for pharmaceutical applications. Tartu, 2015, 156 p.
237. **Triin Laisk-Podar**. Genetic variation as a modulator of susceptibility to female infertility and a source for potential biomarkers. Tartu, 2015, 155 p.
238. **Mailis Tõnisson**. Clinical picture and biochemical changes in blood in children with acute alcohol intoxication. Tartu, 2015, 100 p.
239. **Kadri Tamme**. High volume haemodiafiltration in treatment of severe sepsis – impact on pharmacokinetics of antibiotics and inflammatory response. Tartu, 2015, 133 p.

240. **Kai Part**. Sexual health of young people in Estonia in a social context: the role of school-based sexuality education and youth-friendly counseling services. Tartu, 2015, 203 p.
241. **Urve Paaver**. New perspectives for the amorphization and physical stabilization of poorly water-soluble drugs and understanding their dissolution behavior. Tartu, 2015, 139 p.
242. **Aleksandr Peet**. Intrauterine and postnatal growth in children with HLA-conferred susceptibility to type 1 diabetes. Tartu. 2015, 146 p.
243. **Piret Mitt**. Healthcare-associated infections in Estonia – epidemiology and surveillance of bloodstream and surgical site infections. Tartu, 2015, 145 p.