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Institute of Clinical Medicine, University of Tartu, Estonia

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This PhD thesis is based on the following original research publications:

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II. **Kasvandik, S**; Saarma, M; Kaart, T; Rooda, I; Velthut-Meikas, A; Ehrenberg, A; Gemzell, K; Lalitkumar, PG; Salumets, A; Peters, M (2019). Uterine fluid proteins for minimally invasive assessment of endometrial receptivity. *Journal of Clinical Endocrinology and Metabolism,* 2019, 105 (1), 219–230.

III. Aghajanova, L; Altmäe, S; **Kasvandik, S**; Salumets, A; Stavreus-Evers, A; C Giudice, L (2016). Stanniocalcin-1 expression in normal human endometrium and dysregulation in endometriosis. *Fertility & Sterility*, 2016, 106 (3), 681–691.

IV. Boggavarapu, NR; Lalitkumar, S; Joshua, V; **Kasvandik, S**; Salumets, A, Lalitkumar, PG; Gemzell-Danielsson, K (2016). Compartmentalized gene expression profiling of receptive endometrium reveals progesterone regulated ENPP3 is differentially expressed and secreted in glycosylated form. *Scientific Reports*, 2016, 6, 33811.

Author's personal contribution:

Study I: Participated in the design of the experiments, performed the proteomics experiments, data analysis, bioinformatics and statistical analyses, wrote the manuscript.

Study II: Participated in the design of the experiments, performed the proteomics experiments, data and statistical analyses, and participated in bioinformatics analyses, wrote the manuscript.

Study III: Participated in performing the experiments, data analysis and statistics.

Study IV: Participated in performing the experiments, data analysis and statistics.

ABBREVIATIONS

Abbreviation		Definition
ART	–	assisted reproductive technologies
AUC	–	area under the curve
CV	–	coefficient of variation
ECM	–	extracellular matrix
EEC	–	endometrial epithelial cell
ENPP3	–	ectonucleotide pyrophosphatase/phosphodiesterase 3
ESC	–	endometrial stromal cell
ecESC	–	ectopic endometrial stromal cell
euESC	–	eutopic endometrial stromal cell
ESE	–	early secretory phase
ESI	–	electrospray ionization
ET	–	embryo transfer
FC	–	fold change
HIF	–	hypoxia-inducible factor
ID	–	inner diameter
IVF	–	*in vitro* fertilization
LC	–	liquid chromatography
LCMD	–	laser capture microdissection
LC/MS/MS	–	liquid chromatography tandem-mass spectrometry
LDH	–	lactate dehydrogenase
LPA	–	apolipoprotein(a)
MSE	–	mid-secretory phase
MS	–	mass spectrometry
MSC	–	mesenchymal stem cell
m/z	–	mass to charge ratio
NNMT	–	nicotinamide N-methyltransferase
PCA	–	principal component analysis
PRM	–	parallel reaction monitoring
PTM	–	post-translational modification
RIF	–	repeated implantation failure
RT	–	retention time
qRT-PCR	–	quantitative real-time polymerase chain reaction
SERPINE2	–	glia-derived nexin
SILAC	–	stable isotope labelling with amino acids in cell culture
STC1	–	stanniocalcin-1
WOI	–	window of implantation

1. INTRODUCTION

High-throughput technologies have greatly advanced research in reproductive biology and provided insights for causes behind reproductive illnesses and novel diagnostic methods to detect them. Compared to nucleic acid-based approaches, the contribution of high-throughput proteomics to this advancement has been substantially modest, partly due to high complexity associated with studying proteins. Nevertheless, proteomic studies are important, because proteins are the main functional output of the genome and changes in the proteome reflect the processes occurring in cells and tissues more directly. Fortunately, the last decade has seen tremendous technological developments in mass spectrometry (MS)-based proteomics, which now enables the measurement of entire expressed proteomes of mammalian cells. Therefore, MS proteomics has matured to provide new insights for the continuous challenges in reproductive biomedicine, such as the incomplete understanding of the molecular causes behind non-infectious reproductive diseases and the low efficiency of assisted reproductive technologies (ART).

Endometriosis is a frequent gynecological disease (~5–10% among women of reproductive age) with still unknown pathogenetic mechanism. The disease is characterized by the spread and survival of endometrial cells outside the uterus, which is associated with a significant drop in the quality of life (pain, infertility, depression) for these women. The surgical diagnostics of endometriosis is also cumbersome which results in delayed time to diagnosis, risks associated with surgery and significantly higher costs for the healthcare system. Studying tissues and blood from endometriosis patients with MS proteomics may provide better understanding behind the causes of this illness and provide new minimally invasive markers for its diagnostics.

In vitro fertilization (IVF) is more and more common practice in the developed world, as family planning is pushed to later years of life. The efficacy of IVF as a medical procedure is surprisingly low of about ~30% that is often caused by repeated implantation failure (RIF) of transferred embryos. As RIF may be related to defects in endometrial receptivity, monitoring of proteomic patterns in the minimally invasively obtainable uterine fluid may offer personalized embryo transfer strategies. Such an approach could lead to reduced emotional distress, time and material costs to the patients and the medical system.

The main goal of the current thesis was to apply contemporary MS proteomics methods to tackle ongoing issues in reproductive medicine, *e.g.* discover proteomic changes in endometrial cells that may be behind development and persistence of endometriosis, and, to determine whether proteins secreted to the uterine fluid enable monitoring of normal and disturbed endometrial receptivity.

2. REVIEW OF THE LITERATURE

2.1 Quantitative mass spectrometry-based proteomics

2.1.1 Introduction to mass spectrometry-based proteomics

Proteomics is commonly regarded as the large-scale study of the full complement of proteins (*i.e.* the proteome) of organelles, cells, tissues, organs, body fluids *etc.*, classically by biochemical methods and nowadays mostly through the use of biological mass spectrometry (MS) (Aebersold and Mann, 2016; Pandey and Mann, 2000). Nevertheless, the subjects for proteomics investigations can also include only a selected number of proteins or single proteins, not only complex samples. As a technology-rich biomolecular discipline, the focus of proteomic research centers on both biological insight and the technical advancement of analyzing proteomes.

There are three principal ways for approaching the study of proteomes: (1) on the level of peptides (bottom-up), (2) on the level of large proteolytic fragments (middle-down) and (3) on the level of the intact protein (top-down) (Figure 1). The first approach, bottom-up proteomics, starts out by digesting the proteome, usually under denaturing conditions with the aid of specific proteases (most notably trypsin, which cleaves after lysines and arginines) into individual peptides (Aebersold and Mann, 2003). This renders the proteome conveniently accessible for commonly used methods in analytical separation and detection. Consequently, peptides are separated with liquid chromatography (LC), ionized and directed to a mass detector where the mass to charge (m/z) ratios of their molecular ions and/or fragments are measured (Aebersold and Mann, 2016; Pandey and Mann, 2000). In contrast, top-down proteomics tries to separate and measure the proteins in their intact forms, bypassing the digestion step of the bottom-up workflow (Kelleher, 2004). The third approach, termed middle-down proteomics, is a combination of both bottom-up and top-down approaches, where the proteins are cleaved with minimal proteolysis into larger than peptide fragments and analyzed as such (Taverna et al., 2007).

By avoiding digestion with proteases, the top-down approach provides theoretically the most comprehensive avenue for studying proteomes, as its workflow does not cause any loss of information on the composition of different proteoforms that a complex protein mixture may contain (Smith et al., 2013). However, the complexity of the human proteome has been estimated to be up to ~6 billion different protein species, which arises from alternative splicing, single amino acid polymorphisms (arising from single nucleotide polymorphisms, SNPs) and post-translational modifications (PTMs) of the ~20,000 expressed genes in the genome (Ponomarenko et al., 2016). In addition to the analyte complexity, protein solubilization and separation is less effective than that of peptides. Also, protein signals are diluted due to wide isotopic and charge distributions and not all MS instruments are able to sensitively scan large proteins (Toby et al., 2016). These analytical challenges currently limit the top-down approach to simplified protein

mixtures (*e.g.* purified complexes, soluble and low-molecular-weight fractions of proteomes)(Cheon et al., 2016), thus, further technological improvements are still needed to expand its applications to proteome-wide scale.

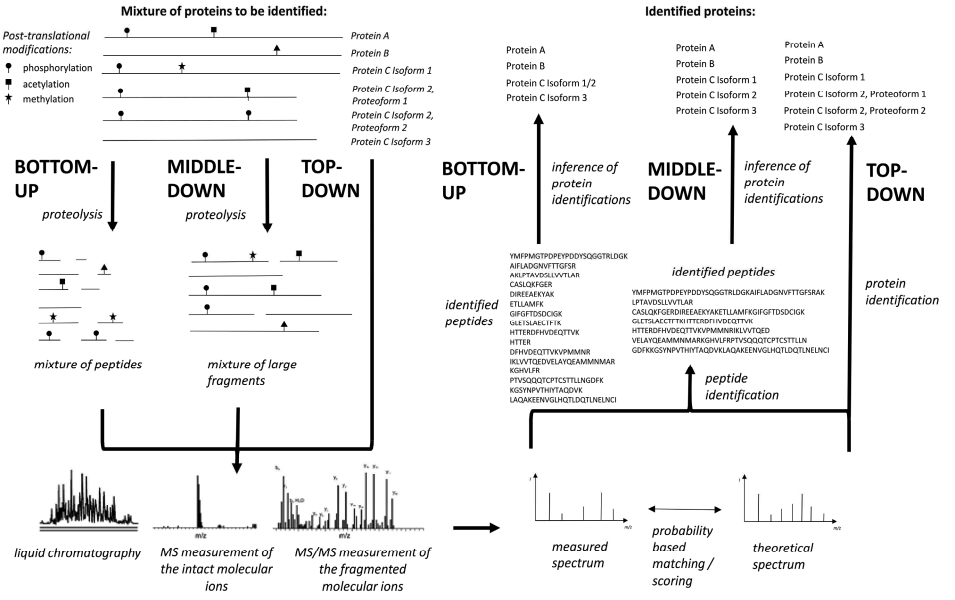


Figure 1. Overview of the MS-based proteomics approaches for the identification of proteins in proteomes: peptide ('bottom-up'), large proteolytic fragment ('middle-down') and protein ('top-down') level identification. All three approaches differ in the sample preparation prior to LC/MS/MS and in the level of detail of the results, but involve chromatographic separation and tandem-MS (MS/MS) data acquisition steps.

On the other hand, the bottom-up approach is a well-established framework for proteomics applications and its proteome coverage has now nearly reached the entire expressed proteome (*i.e.* covering most of the annotated genes while not accounting for proteoform diversity) of eukaryotic cells (Mann et al., 2013). As contrasted to direct protein analysis above, it owes its success to the analytical accessibility of its substrates, *i.e.* to peptides (0.7–3 kDa). Peptides have much better solubility compared to full-length proteins and display excellent separation, ionization and fragmentation behavior in LC/MS/MS. Vast number of workflows for bottom-up approach have been implemented, either for qualitative or quantitative analyses, focusing on PTMs (*e.g.* phosphorylation, ubiquitinylation *etc.*) or unmodified proteins, with a growing number of software solutions to analyze the data from raw spectra to final statistical and bioinformatical analysis (Cox and Mann, 2008; MacLean et al., 2010; Tsiamis et al., 2019). Unfortunately, as illustrated in Figure 1, cleavage of proteins into peptides creates potential loss of information, known as the protein inference problem (Nesvizhskii and Aebersold, 2005). This means that based on identified peptides it is sometimes complicated, if not impossible, to infer which exact protein or proteins were

found in the sample, as the presence of significant sequence overlap/identity and missing peptide sequences make it difficult to prove/disprove one or the other. This has more relevance to human samples where multiple protein proteoforms can be produced due to alternative splicing. As MS instruments are continuously improved and subject to ongoing advancements, higher and higher sequence coverages can be expected to be obtained. Thus, to a certain extent reducing this conundrum in shotgun experiments. Utilizing targeted MS for validation experiments, unique sequences can be chosen avoiding the inference problem altogether, with detection specificities outperforming classical methods such as immunoassays (Aebersold et al., 2013).

To overcome limitations set by the bottom-up approach for detecting different co-occurring PTM patterns, middle-down approach has demonstrated to be an alternative strategy, especially for the analysis of variably modified histone tails, monoclonal antibodies and branched ubiquitin chains (Cristobal et al., 2017). Nevertheless, middle-down approach requires digestion optimization to produce sequences of desired length (~3–10 kDa) and currently no commercially available protease works for all proteins.

2.1.2 Instrument platforms used in MS-based proteomics

Modern MS-based proteomics instruments are intricate machines carrying out peptide/protein separation, analyte ionization, precursor isolation/fragmentation (for tandem MS or MS/MS), *m/z* measurements and data collection. A basic scheme of an LC/MS/MS instrumentation is made up of an LC-unit, ion source and MS instrument with multiple mass analyzers and a fragmentation section(s) (Figure 2). It is also possible to bypass the LC-separation step by directly infusing the samples into the MS.

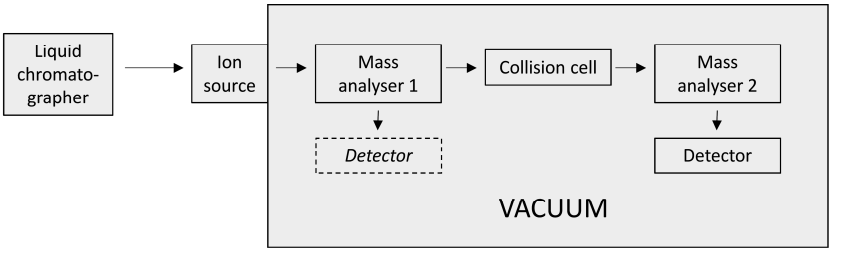


Figure 2. General scheme of an LC/MS/MS instrument. Note, the first mass analyzer can also sometimes perform ion detection (*e.g.* in ion traps), but often functions only as an *m/z* filter (*e.g.* in quadrupole tandem instruments).

The emergence of MS-based proteomics was largely made possible by the introduction of soft ionization techniques, namely matrix-assisted laser desorption/ionization (MALDI) (Karas and Hillenkamp, 1988; Tanaka et al., 1988) and electrospray ionization (ESI) (Fenn et al., 1989). Both of these methods enabled

effective ionization of peptides and proteins without fragmenting their molecular structures. In MALDI, ions are created by transferring laser energy to ultraviolet-absorbing matrix molecules (mostly organic acids, *e.g.* sinapinic acid) which then impart a charge to peptides/proteins mixed with the matrix. In ESI, analytes in the liquid phase are ionized by creating a strong electric field between a capillary tip (*e.g.* coming from the LC) and the MS inlet. This leads to charge accumulation on the liquid surface, expulsion of charged droplets and evaporation or ejection of ionized peptides and proteins (Wilm, 2011). As a general rule, peptides and proteins become multiply charged during ESI, which makes them amenable to the *m/z* scan ranges of popularly used MS analyzers (*i.e.* orbitraps, ion traps, time-of-flight analyzers). Secondly, the ease by which ESI can be coupled to the LC and thereby combine separation and MS measurements in real-time, has made it the mainstay ionization method in MS-proteomics.

LC plays another essential role in high-throughput proteomics by simplifying the complex mixtures introduced to the MS. Unlike in small molecule applications, miniaturization of the LC system flow-rates has been strongly favored in proteomics due to the sensitivity gain for limited sample amounts (Mitulovic and Mechtler, 2006). Reducing the inner diameter (ID) of columns squarely increases the concentration of analytes in the solution, *e.g.* going from 4.6 mm ID column (a regular analytical LC) to a 75 µm ID column (a common nano-LC size) results in theoretical sensitivity gain of >3,700-fold. For peptide and protein analysis, packed and monolithic reversed phase (RP) stationary phases are preferred due to their great resolving capabilities and compatibility with both ESI and MS (Fanali et al., 2013). However, for comprehensive PTM analyses (*e.g.* glycosylation) dedicated column materials should be preferred that enable better retention and separation than RP for those chemically distinct peptide species.

Nearly all MS detectors (Table 1) can be used for proteomic analyses, yet for most discovery type experiments high resolving power and fast scanning speeds are required. Modern instruments mainly utilize orbitrap and time-of-flight (TOF) as detectors, and, quadrupole or ion trap for precursor filtering. TOF analyzers measure the flight time of ions that are dependent on their *m/z* values (Hoffmann and Stroobant, 2007). In contrast, Orbitrap analyzer records harmonic oscillations of ions along a spindle-shaped electrode, where the frequency of oscillations is related to their *m/z* values (Olsen et al., 2005).

Table 1. Common MS analyzers and their properties (reproduced from (Hoffmann and Stroobant, 2007), (Barner-Kowollik, 2012) and vendor specifications).

	Magnetic sector	**Quadrupole**	**3D ion trap**	**Linear ion trap**	**Time-of-flight**	**Ion cyclotron resonance**	**Orbitrap**
Ion property for *m/z* measurement	Ion deflection in a magnetic field	Passage of an ion through oscillating electric fields specific to an *m/z* value	Applied voltage frequency for ion ejection from analyzer	Applied voltage frequency for ion ejection from analyzer	Ion flight time through analyzer	Ion cyclotron frequency	Ion axial oscillation frequency along a spindle-shaped electrode
Resolution	10^4 (10^5)	1×10^3 (10^4)	4×10^3 (10^4)	5×10^3 (3×10^4)	1×10^4 (4×10^4)	6×10^5 * (10^7)	2.4×10^5* (10^6)
Mass accuracy	<5 ppm	<0.5 Da	<0.5 Da	<0.5 Da	<5 ppm	<1 ppm	<2 ppm
Linear dynamic range	10^9	10^7	10^2–10^3	10^3–10^4**	10^6	10^3–10^4**	10^3–10^4**
Advantages	High resolution and mass accuracy, superior dynamic range.	Tolerant of high-pressures, low cost, high sensitivity.	Multiple tandem MS (MS^n) capability, cheap cost and maintenance.	Multiple tandem MS (MS^n) capability, cheap cost and maintenance, fast scanning.	Fast scanning speeds, high mass-ranges and accuracy, low cost.	Ultra-high resolution and mass accuracy.	High resolution and mass accuracy, fast scanning, less complex than magnetic sectors and cyclotron resonance instruments.
Disadvantages	Slow scanning, size of the instrumentation, expensive.	Low resolution and mass accuracy.	Low resolution and mass accuracy.	Low resolution and mass accuracy.	Lower sensitivity, limited resolution.	Expensive cost and maintenance, sharp drop of resolution with increasing *m/z*.	Drop of resolution and sensitivity with increasing *m/z*.
Example commercial instrument	Spectro MS® (Spectro)	6470A Triple Quadrupole (Agilent)	LCQ® (Thermo Fisher Scientific)	LTQ Velos Pro® (Thermo Fisher Scientific)	TripleTOF® (Sciex)	solariX® (Bruker)	Fusion Lumos® (Thermo Fisher Scientific)

** resolution at 400 m/z at a scan rate of 1 Hz ** up to 10^6 between spectra*

2.1.3 MS/MS data acquisition strategies

The qualitative and quantitative nature of proteomic data are not only determined by the wide selection of analyzers available, but also by the chosen strategy to collect the data on a tandem-MS instrument. Data acquisition mode refers to the specific way the MS/MS system is directed to record data for peptide/protein identification and quantification. There are mainly three different approaches in contemporary proteomics: (1) data-dependent (DDA), (2) targeted and (3) data-independent acquisition (DIA) (Schubert et al., 2017). After the spread of high-performance MS/MS instruments, techniques such as peptide fingerprinting (protein identification based on solely intact peptide masses) (Henzel et al., 1993) have now largely become obsolete.

Since its inception, the DDA mode (Aebersold and Mann, 2003) has remained popular, especially for discovery proteomics. It operates on a simple decision tree of intensity-based selection of peptides for MS/MS. This decision tree is frequently coupled with a feature called dynamic exclusion, where precursors selected for fragmentation are put on a time-limited hold to avoid repeated analyses of the same peaks (Zhang et al., 2009). DDA is an excellent method for discovery-oriented investigations as its breadth of detection still surpasses others, yet due to its somewhat stochastic nature in peak selection, undersampling and low reproducibility of trace-level proteins can occur (Hu et al., 2016). Nevertheless, transfer of peptide identifications based on accurate mass and retention time (RT) have been implemented to improve data coverage in DDA experiments (Tyanova et al., 2016). Recently, the dynamic range and depth of proteome coverage was also improved by the introduction of the so-called BoxCar methods, where ions from multiple narrow *m/z* ranges are collected by differing lengths of time before their measurement in the analyzer (Meier et al., 2018).

Targeted MS analysis in the form of selected, multiple and parallel reaction monitoring (SRM, MRM and PRM, respectively) has become the gold-standard in high sensitivity and specificity peptide/protein measurements (Marx, 2013). Whereas DDA is mostly intended for generating hypotheses, targeted MS is used for confirming them. This, however, entails that prior information about specific proteins is known (*e.g.* from a discovery experiment, mRNA levels *etc.*) or hypothesized (*e.g.* based on literature), followed by method development where RTs and quantitatively useful fragments of peptides (synthetic or from DDA data) are determined (MacLean et al., 2010). As targeted MS approach measures only a limited number of peptides repeatedly and without interference, it attains very high quantitative precision and sensitivity (Marx, 2013). Also, its specificity is considered superior to classical confirmatory methods like Western blot or ELISA (Aebersold et al., 2013). Overall, targeted approaches are excellent for development of specific assays.

DIA, conceptually described more than a decade ago (Venable et al., 2004), has only recently been ‘put to practice, especially after the introduction of software solutions to tackle the complex nature of DIA datasets (Searle et al., 2018; Ting et al., 2017; Tsou et al., 2015). In DIA, MS/MS fragmentation is carried out

in small windows (*i.e.* Δ20 *m/z*) of a mass range (*i.e. m/z* 500–900), thereby collecting data for virtually all peptides in the selected range. By the virtue of narrowing the peptide detection windows, DIA offers greater sensitivity than DDA, nearing that of SRM/MRM – a crucial feature for improving the analytical dynamic range for samples with wide intra-sample analyte concentration ranges (Lin et al., 2018). The challenges with DIA are still associated with the complex spectra that are sometimes cumbersome to interpret exhaustively, but an ongoing effort is being made to overcome these hurdles (Hu et al., 2016).

2.1.4 Experimental design strategies for quantitative proteomics

Raw MS signal is not inherently an accurate measure of the absolute quantity of an analyte present in a sample, as it varies due to many factors such as individual ionization efficiencies of peptides and the presence of co-ionizing substances (Aebersold and Mann, 2003). Therefore, most quantitative studies in proteomics are done on a relative scale, *i.e.* comparing the change in MS signals from sample-to-sample. Accurate absolute quantification of individual proteins can be achieved using spiked-in known amounts of isotopically labelled synthetic peptides (Bantscheff et al., 2012). However, for practical reasons this is not feasible on a proteome-wide scale, and methods that rely on the averaging effect of multiple peptide ionization efficiencies onto the protein level have been devised (Bantscheff et al., 2012). Nevertheless, this is not always accurate and is to be used only when rough estimations are sufficient for study purposes.

Workflows in quantitative proteomics can broadly be categorized into two main branches: (1) label-based, and (2) label-free (Figure 3). In label-based approaches one or more of the samples are modified by incorporation of either stable isotopes (*e.g.* ^{13}C, ^{15}N) or chemical adducts (*e.g.* variably isotope-coded acyl groups). Differently labelled samples are then mixed and further processed/ analyzed as one sample (Aebersold and Mann, 2003). In contrast, with label-free proteomics approaches all samples are processed separately throughout all steps (Bantscheff et al., 2012).

Most common of all the label-based methods are the stable isotope labelling with amino acids in cell culture (SILAC) and the isobaric tagging of peptides (Bakalarski and Kirkpatrick, 2016). In SILAC, one of the samples is grown in the presence of stable isotope-labelled ('heavy') amino acids, while the other uses natural ('light') counterparts (Ong et al., 2002). Commonly, either heavy or medium heavy lysine ($^{13}C_6$ or $^{13}C_6{}^{15}N_2$-containing), heavy arginine ($^{13}C_6$ or $^{13}C_6{}^{15}N_4$-containing) or both are used. Such a selection of amino acids is desirable, as in the case of trypsin (cleavage after Lys and Arg) or Lys-C (cleavage after Lys) all peptides will be labelled with the respective amino acid (except for C-terminal peptides that do not have Lys or Arg in their termini). After labelling, the samples can be combined (Figure 3) and processed together eliminating any technical variability that can ensue from separated handling, and to reduce analysis time

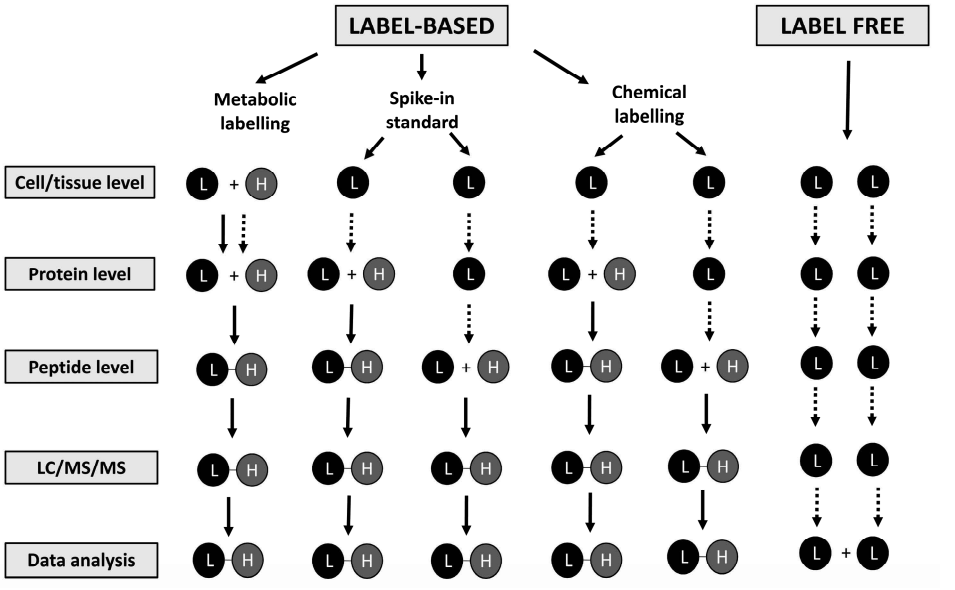


Figure 3. Different quantitative proteomics workflows. In case of metabolic labelling, light (L) and heavy (H) samples can be combined either on cell or protein level, depending on a particular experimental setup. Note, dashed arrows indicate steps where technical variation and/or bias can occur.

due to multiplexing. Quantitative data will be read out from the relative intensities of MS-resolved and mass-separated peaks of the heavy and light peptides, both of which behave identically during chromatography and ionization (Geiger et al., 2011). The limited drawback of SILAC is that it dilutes the signal from the same peptide across labels and reduces peptide identification rates by increasing number of peaks in the MS spectra (contributing to the missing values problem mentioned before) (Bakalarski and Kirkpatrick, 2016). SILAC in its original form can only be carried out on limited number of samples (*i.e.* using light, medium heavy and heavy amino acids). Nevertheless, it can be extended to an unlimited number of samples by using one of the samples as a spike-in reference to essentially derive ratios of ratios (Geiger et al., 2011) – the only cost being increased measurement time and repeated MS/MS sampling of a reference. The spike-in approach can be advantageous when working with material for which no labelling would be conceivable (*e.g.* clinical samples like human tissues), instead the standard can be made representative of the material by combining several metabolically labeled cell lines (termed 'Super-SILAC') (Geiger et al., 2010; Shenoy and Geiger, 2015).

Isobaric tagging of peptides (*e.g.* by methods such as iTRAQ or TMT) introduces multiplexing of samples by utilizing intelligently designed chemical reagents (Bachor et al., 2019). These reagents have identical MS-level masses, but undergo cleavage under MS/MS yielding fragments with different masses that are dependent on the reagent used for a specific sample (Ross et al., 2004;

Thompson et al., 2003). Relative intensities of those reporter fragment ions directly reflect the protein abundances across samples. As at the MS1-level masses are merged, iTRAQ and TMT do not dilute the MS signal, thus, enabling better sensitivity in addition to the high-throughput achieved from multiplexing. Also, coverage for quantitative data is enhanced and occurrences of missing values are reduced because peptides from all samples in an experiment are analyzed by a single comprehensive MS/MS event. Isobaric tagging has been described up to 18-plex by using triplex SILAC with 6-plex TMT (Dephoure and Gygi, 2012), but the current availability of 11-plex reagents can theoretically enable a 33-plex version. MS/MS-level quantitative analysis can, however, suffer from dynamic range compression issues leading to quantitative inaccuracies if co-eluting peptides happen to be co-isolated. For such cases only gas phase separation (Wenger et al., 2011) or MS/MS/MS analysis can rectify compression distortions (Ting et al., 2011).

A promising, however still in proof-of-principle stage label-based method, termed NeuCode has recently been described that combines the benefits of both SILAC and isobaric tagging (Hebert et al., 2013). The approach exploits very small mass differences of below mDa, arising from neutron mass defect of different elements (Hebert et al., 2013). Such mass differences are achieved by using differentially labelled ^{15}N and ^{13}C amino acids, resulting in isobaric MS and MS/MS signals at low resolution, however quantitative read-outs of the iso-topologues are achieved using ultra-high resolution scans MS1 (*i.e.* around 500,000 at 400 *m/z).* Nevertheless, the approach requires high-performance instruments capable of fast ultra-high resolution scanning (such instruments are currently not yet widespread, mainly due to their high cost), and requires developments in the understanding of the peak merging effect of closely spaced high abundant isotopologues (Bakalarski and Kirkpatrick, 2016).

Technically the simplest way to measure protein abundance is via the use of label-free analysis (Figure 3), which processes samples separately and where proteins are compared based on their cumulative LC/MS/MS signals (*e.g.* number of scans per protein or area under the curve (AUC) of peptides associated with a protein) (Wang et al., 2019). Evidently, label-free offers no multiplexing and potentially adds the highest degree of technical variability to the data compared to label-based methods. Yet, recent studies have shown that modern instruments can offer comparable quality to that achievable with SILAC (Tebbe et al., 2015). This is further facilitated by advanced normalization techniques (Cox et al., 2014) and statistical modeling that incorporates the usage of multiple MS-data features (Clough et al., 2012). Label-free is particularly suitable for clinical samples where suitable standards are not easily available or cannot be produced (Cox et al., 2014). Another advantage is a higher dynamic range (2–3 orders of magnitude) compared to labeled methods (1–2 orders of magnitude), nevertheless, the latter captures small changes in relative abundance with more accuracy and precision (Schulze and Usadel, 2010).

The final choice of optimal quantitative strategy will come down to the specific needs of a particular experiment and experimenter. Factors such as the

availability of standards, specific instruments in the laboratory, number of samples, magnitude of differences of interest, required robustness of measurements and available budget will have to be weighed against each other to determine the best-suited approach.

2.2 Emerging role of proteomics in reproductive biology

The research in reproductive biology focuses mainly on the male and female reproductive systems and on the embryo. The emphasis is on understanding the underlying biology, elucidating causes behind infertility (*e.g.* dysfunction of sperm, oocytes, embryos or endometrial functioning), finding new diagnostic biomarkers and improving assisted reproductive technologies (ART). More advanced technologies are required, as the prevalence of infertility in developed countries is growing and the current ART technologies have poor efficacy of around 30%, rendering the overall practice relatively inefficient and costly for the healthcare system (Kupka et al., 2014).

Before the completion of the Human Genome Project and the advent of sequencing technologies, most of the research in molecular reproductive medicine was led by hypothesis testing with classical biochemistry and molecular biology tools. During the last decades, use of hypothesis-free omics-technologies has shown a growing trend (Altmae et al., 2014). Application and studies involving proteomics have nevertheless been relatively modest, especially compared to genomics and transcriptomics (Figure 4). This can be attributed to the complexity and limited availability of proteomics technologies, but also to the dynamic range challenges that proteomes pose compared to genomes and transcriptomes (Zubarev, 2013). For example, where mRNA levels in the cell

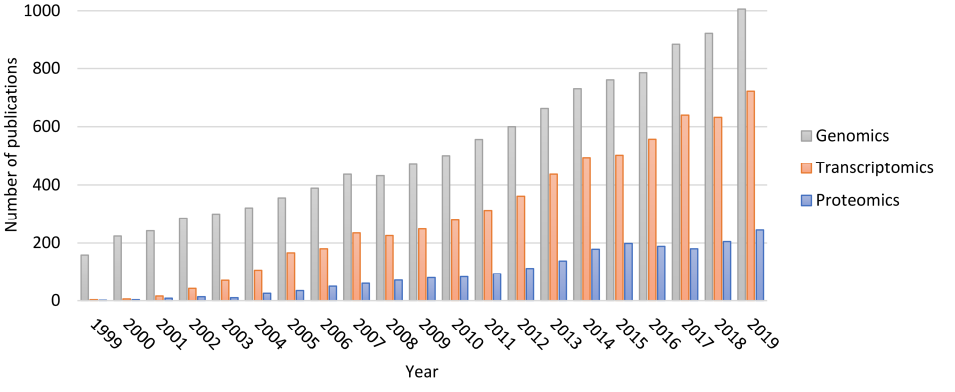


Figure 4. Number of PubMed listed articles from 1999–2019 related to reproductive sciences and different omics-methods. Data were retrieved by searching for reproduction ("*reproduction*", "*endometrium*", "*sperm*", "*oocyte*", "*embryo*", "*ovaries*", "*testis*") and omics- ("*genome*", "*genomics*" / "*microarray*", "*rna seq*", "*transcriptome*" / "*proteomics*", "*proteome*") related keywords in article abstracts.

may span only three to four orders of magnitude, protein abundance varies on a scale of at least seven orders of magnitude (up to ten orders of magnitude for blood) and even more given the differing signal responses of peptides after protein digestion. Nevertheless, proteomics is expected to provide the most novel findings for the field in the upcoming decades (Altmae et al., 2014).

2.3 Endometrial function in health and pathology

2.3.1 Role of endometrial cells in uterine functioning and embryo implantation

Endometrium as the innermost layer of the uterus plays a pivotal role in the attachment of the developing embryo to the maternal organism, and, in the initiation of pregnancy. During the menstrual cycle, successful implantation is considered possible only in a short period of time, known as the window of implantation (WOI) (Harper, 1992). WOI usually starts on cycle day 19 or 20 and lasts about 4–5 days. The execution of the attachment can only happen when the maternal conditions are optimal for accepting the developing blastocyst, therefore, the endometrium can be considered as a gatekeeper (Macklon and Brosens, 2014).

Endometrial tissue is mainly composed of glandular and luminal epithelial cells and stromal cells (making up the functional layer of the endometrium), but includes also somatic stem cells, vascular endothelial, blood and immune cells (Jimenez-Ayala et al., 2008; Lee et al., 2011a). Histologically, the glandular cells form tube-like structures that are located inside a vascular stroma (Figure 5A), rest of the cellular interface to the uterine lumen is made up of luminal epithelial cells (Jimenez-Ayala et al., 2008). This functional layer of endometrium is responsive to ovarian hormones (estrogen and progesterone), regenerating and (unless successful fertilization has taken place) shedding every menstrual cycle, lasting on average 28 days (Figure 5B). During the proliferative phase of the menstrual cycle, estrogen causes the endometrial cells to proliferate, whereas in the secretory phase progesterone arrests mitotic activity and triggers differentiation.

Endometrial epithelial cells (EECs) are the first to make a contact with the blastocyst during the implantation process. In humans, this can only happen successfully about 6–10 days after ovulation and implantation attempts outside this timeframe lead to its failure, demonstrating that endometrial receptivity is under tight maternal control (Aplin and Ruane, 2017). In later stages of implantation, the EECs are displaced and the embryo, guided by trophoblasts, invades into the stroma, until the epithelium eventually covers it fully. The process of initial apposition, attachment and invasion through the luminal epithelium has been suggested to involve multiple cell surface glycoproteins, including mucins (Aplin et al., 2001), trophinin (Sugihara et al., 2007), dystroglycan (Heng et al., 2015) and several integrins (Aplin and Ruane, 2017).

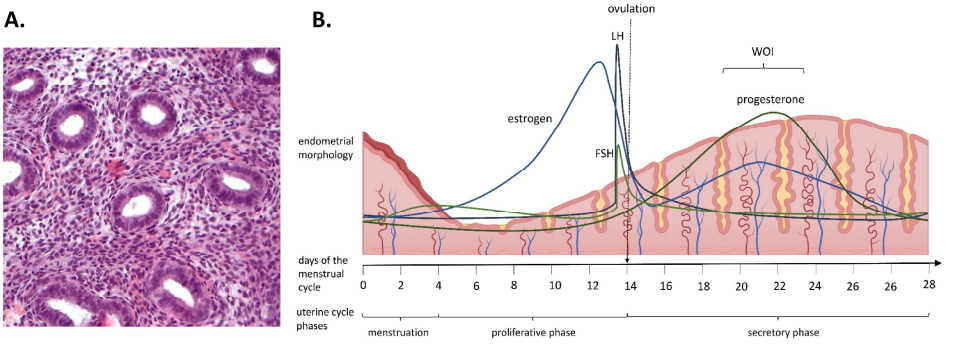


Figure 5. A. Cross-sectional micrograph of an early secretory phase endometrium showing endometrial glands (tubular structures) and the surrounding stroma. Image courtesy of Triin Laisk from the Institute of Clinical Medicine, University of Tartu. **B.** Endometrial tissue and its changes throughout the menstrual cycle. Concentration curves of circulating hormones have been indicated. Note, that the sharp rise of gonadotropins FSH and LH trigger ovulation in the beginning of the secretory phase. Image produced using the BioRender App (https://app.biorender.com/). FSH – follicle stimulating hormone, LH – luteinizing hormone, WOI – window of implantation.

Endometrial stromal cells (ESCs) are mostly of fibroblastic nature and morphology. Towards the end of the proliferative and throughout the secretory phase, they markedly expand in volume in response to progesterone stimulus that triggers a whole cascade of changes in the endometrium known as decidualization. The increased volume of ESCs is simultaneously accompanied by acquiring epithelial-like morphology and an increase in ribosomes, mitochondria, residual bodies, glycogen and lipid droplets in the cytoplasm. Proteomic profiles of these decidual stromal cells are also markedly changed (Paule et al., 2011), leading to the secretion of multiple cytokines, growth factors and extracellular matrix (ECM) proteins. These processes are required for ESCs to establish optimal interaction with the embryo, by recognition and selection processes of the embryo, and to stimulate the activity of vascular and immune cells within the endometrial tissue (Zhu et al., 2014a).

The endometrium also houses stem cells (Chan et al., 2004), that are responsible for the regeneration of the functional layer after each menstruation (Gargett et al., 2016). Endometrial stem cells are usually found, albeit scarcely, near blood vessels in the basal and functional layer. Despite not being clear yet whether epithelial and stromal cells derive from different stem cells, they are hypothesized to belong to bone marrow-derived stem cells and their involvement in several uterine pathologies (*e.g.* endometriosis) is considered relevant (Djokovic and Calhaz-Jorge, 2014).

2.3.2 Assessment of normal endometrial receptivity

The WOI encompasses the synchronization of spatiotemporal functions of endometrial cells, including changes in their secretome, eventually rendering the tissue and surrounding environment receptive and supportive to the embryo implantation (Bhusane et al., 2016). Yet, the acquisition of the receptive state in the endometrium is not considered to be an all-or-none event, but rather a graduated phenomenon with more or less optimal outcomes for implantation and – of pregnancy (Lessey and Young, 2019).

Since the 1950s, tissue histology criteria set forward by Noyes *et al* have been used to assess endometrial receptivity of biopsies obtained from the endometrium (Noyes et al., 1950). These criteria correlate the morphological changes in glands and stroma (shape of glandular structures, signs of mitotic activity, presence of subnuclear vacuoles, stromal edema, amount of luminal secretions *etc.*) to the cycle day of the menstrual cycle. However, the accuracy and precision of this approach are poor, subject to interobserver variability and non-morphological receptivity defects in the endometrium remain undetected (Murray et al., 2004). Thus, significant effort has been made to identify receptivity markers at the molecular level.

Numerous studies have reported multiple different proteins associated with the opening of the WOI and their disrupted expression in infertility. There is a significant amount of evidence that aberrant progesterone signaling, or progesterone resistance, is often present in different conditions leading to impaired endometrial receptivity and infertility (Choi et al., 2016; Fox et al., 2016; Joshi et al., 2017; Lessey and Young, 2019). Progesterone exerts numerous downstream effects in the endometrium (Large and DeMayo, 2012), including down-regulation of its own expression and that of estrogen receptor alpha (ESR1), and the cross talk with multiple transcription factors (*e.g.* IHH, HOXA10, HAND2, FOXO1, STAT3, GATA2, SOX17) (Marquardt et al., 2019). One of the hallmarks of progesterone resistance is the persistently elevated level of its nuclear receptor (PGR) at the time of implantation in the uterus (Fox et al., 2016). A wider consequence of progesterone resistance is increased inflammation through unbalanced estrogen signaling – phenomenon which is thought have significance also for the symptoms of endometriosis (Marquardt et al., 2019). Why endometrial progesterone signaling becomes abnormal in some cases is not precisely clear, however, at least in women with endometriosis sirtuin-1 (SIRT1) and B-cell lymphoma 6 protein (BCL6) may be important, as they increase in activity in response to inflammatory stimuli, and, in complex with each other interfere with progesterone target gene expression (Lessey and Young, 2019; Yoo et al., 2017).

Another set of single markers described to correlate with the WOI opening are various integrins (especially integrin αvβ3), L-selectin ligand, matrix metalloproteinases, E-cadherin, pregnancy-associated endometrial alpha 2-globulin (alpha-2 PEG), luteinizing hormone/choriogonadotropin receptor (LHCGR), leukemia inhibitory factor (LIF), macrophage colony-stimulating factor (CSF1), transcription factor HOXA10 and vascular endothelial growth factor A (VEGFA)

(Craciunas et al., 2019). Nevertheless, none of the single markers has produced convincing evidence for clinical use, either lacking in sensitivity and/or specificity (Craciunas et al., 2019). Higher diagnostic power can be achieved by monitoring simultaneously the activity of multiple markers, *i.e.* with biomarker panels, such as the 248-gene transcriptomic test 'Endometrial receptivity array' (ERA® test) (Diaz-Gimeno et al., 2011a).

2.3.3 Assessment of pathological endometrial receptivity

The assessment of attainment of a sufficiently receptive endometrium may provide greatest benefit for a group of IVF patients who suffer from repeated implantation failure (RIF) without any other known reproductive problems (endometriosis, endometrial abnormalities, premature ovarian insufficiency, polycystic ovarian syndrome *etc.*). RIF diagnosis is considered when at least three implantation failures with good-quality embryo transfers have occurred or conception was not achieved after a transfer of at least ten good-quality IVF embryos (Sebastian-Leon et al., 2018). Subsequently, endometrial factor remains as the most suspected, but not finitely proven culprit behind those cases of unexplained RIF.

Currently there are two emerging theories for endometrial receptivity-based RIF causes in IVF: a temporally displaced or a disrupted WOI. In case of the displaced WOI, RIF is happening due to asynchrony between the developing blastocyst and the endometrium (Ruiz-Alonso et al., 2013; Ruiz-Alonso et al., 2014). This was deducted based on the fact that personalized embryo transfer (ET) time in RIF women lead to an increased number of pregnancies (Patel et al., 2019; Ruiz-Alonso et al., 2013). Other studies have also reported that women undergoing frozen ET have higher pregnancy rates if their ET is temporally adjusted after a non-receptive ERA® test result (Rosen et al., 2019; Tan et al., 2018). Although, contradictory reports have also been published, where personalized ETs with ERA® did not improve pregnancy rates in women with good prognosis (*i.e.* with 0–2 previous frozen ETs), even though ERA® reported 64% of these women to be pre- or -post-receptive (Bassil et al., 2018). Nearly all the studies call for larger prospective studies to determine whether ERA® provides a meaningful result for personalizing ET.

On the other hand, as the ERA® test does not appear to help all RIF patients, it is possible that another subpopulation of RIF patients suffers rather from a generally disrupted gene expression in the endometrium than just from a temporally displaced WOI. As the ERA® test was originally derived based on only healthy fertile women, Koot *et al* theorized that a different transcriptomic profile based on direct analysis of RIF women (with suspected endometrial disruptions) and healthy controls might provide a better gene expression signature for RIF diagnostics (Koot et al., 2016; Macklon, 2017). Authors of that study found that RIF women indeed have evidence for altered gene expression in the endometrium (Koot et al., 2016; Macklon, 2017).

To find a common ground between the displaced and disrupted theories for RIF, a recent report put forward that both the displaced and disrupted signatures can be detected at the same time in some RIF patients, while other RIF patients can be uniquely classified either as with a signature indicative of a displaced or a disrupted WOI (Sebastian-Leon et al., 2018). Consequently, Sebastian-Leon *et al* proposed a new RIF taxonomy that stratifies RIF patients into four distinct categories having different clinical implications for treatment (Figure 6).

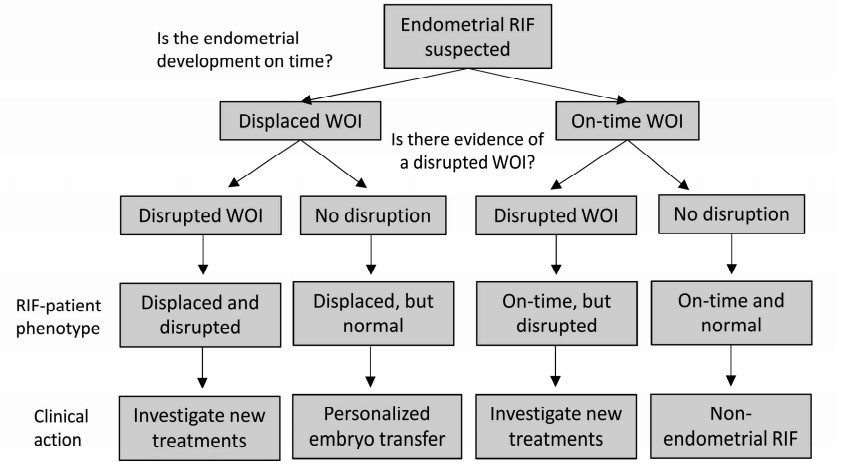


Figure 6. Recurrent implantation failure taxonomy as suggested by Sebastian-Leon *et al*, 2018. Given two potential molecular causes and their specific molecular (transcriptomic) patterns in the endometrium for RIF (displaced or disrupted WOI), patients can be categorized into four groups, each of which should be clinically addressed as appropriate. Note, that both causes can simultaneously be present. Figure reproduced and modified from Sebastian-Leon *et al*, 2018.

2.3.4 Uterine fluid as an indicator of endometrial health and development

The fluid of the upper female reproductive tract (*i.e.* the fallopian tubes and the uterus) plays an essential role in embryo development and implantation, as it is the medium and buffer where the early embryo develops and finds its way to the correct implantation site in the uterine wall (Hu and Yu, 2017). Embryo implantation fails in the absence of endometrial glands – the main secretory source of the fluid in the uterus (Filant and Spencer, 2013; Gray et al., 2002). In addition, disruption of uterine fluid homeostasis can cause incorrect embryo implantation and pregnancy loss (Zhang et al., 2015). Uterine liquid environment is also necessary for sperm transit prior to fertilization.

The content of uterine fluid is made up of secreted molecules from the cells lining the uterine cavity, *i.e.* primarily from endometrial epithelial cells, but also from immune and vascular cells and extracellular vesicles released by various

cell types present in the endometrium (Lee et al., 2015; Ng et al., 2013). In addition, soluble contents from the fallopian tubes and the cervicovaginal region may partially end up and mix with the fluid in the uterus (Casado-Vela et al., 2009). Steroid hormone dependent secretion of small molecules, such as simple carbohydrates, amino acids, metabolic intermediates (*e.g.* pyruvate, lactate), antioxidants, electrolytes, lipids and lipid hormones in uterine fluid are all necessary to sustain embryo viability and development until a dedicated blood supply has been established for an implanted embryo (Bhusane et al., 2016; Hu and Yu, 2017). Signaling proteohormones, such as cytokines, chemokines and growth factors are differentially regulated during the WOI and regulate endometrial function (Berlanga et al., 2011). So far, the entire proteomic diversity in uterine fluid has been estimated to consist of up to a few thousand different proteins (Fitzgerald et al., 2018b; Hannan et al., 2012; Parmar et al., 2008).

Volume of the uterine fluid and its regulation during the peri-implantation timeframe seems to be an important factor for implantation success and for the correct implantation site, as well. Under normal circumstances, fluid volume is decreased prior to embryo attachment to facilitate luminal closure and prevent floating of the embryo in the uterine cavity (Zhang et al., 2017). This process is under the control of progesterone/estrogen balance and in rodent models is disrupted by excessive estrogen (Zhang et al., 2015).

Sampling uterine fluid for endometrial diagnostics has many desirable attributes over taking biopsy samples from endometrial tissue, as currently done for tests such as ERA®. The latter is considered relatively invasive and excludes embryo transfer in the same IVF cycle as the biopsy. However, collecting a uterine lavage or fluid is minimally invasive and does not impair pregnancy rates when performed in the same cycle for IVF and ET (Berkkanoglu et al., 2004; Boomsma et al., 2009a; Olivennes et al., 2003; van der Gaast et al., 2003). Efforts have been made to correlate transcriptomic patterns of uterine fluid to the changing receptivity status of the endometrium with results showing that it indeed similarly predicts opening of the WOI (Chan et al., 2013). Nevertheless, the need for RNA amplification and the use of mRNA sequencing renders the process potentially less reproducible and less cost-efficient than a simplified rapid ELISA-type of assay measuring proteins in the fluid. Therefore, development of protein-based marker panels, which could be used with high throughput in the same cycle with IVF-ET, is highly desired for clinical use.

Studies on uterine fluid proteins have reported many proteins to be implicated in endometrial receptivity (Table 2). Unfortunately, none of the proposed markers have made into clinical diagnostic use and most studies have been exploratory without proper follow-up validation or the markers have had insufficient sensitivity/specificity for diagnostics (Craciunas et al., 2019). Results with the best reported classifier performance have been achieved with uterine fluid LIF measurements, which did appear to discern women with unexplained infertility fairly well from fertile controls (Mikolajczyk et al., 2003). However, other results have not been highly consistent and the value of LIF as a single marker for

Table 2. Overview of uterine fluid studies on endometrial receptivity proteins.

Study	Subjects	Method	Targets	Results	Proposed classifier and its performance
(Halperin et al., 1995)	109 women undergoing IVF-ET	ELISA	hDP 200	Lower levels of hDP 200 in women with successful implantation	hDP 200 <1000 mU/mg, SE=57.1% SP=69.5%
(Ledee-Bataille et al., 2002)	30 women undergoing IVF-ET	ELISA	LIF	Lower LIF ($p=0.001$) at cycle day 26 in women who became pregnant	NA
(Mikolajczyk et al., 2003).	16 fertile, 49 infertile (multiple causes) and 30 women with recurrent miscarriage	ELISA	LIF	Women with idiopathic infertility have lowered LIF levels ($p<0.05$).	LIF <8.23 pg/ml, SE=86.7% SP=100%
(Olivennes et al., 2003)	148 women undergoing IVF-ET	ELISA	LIF	LIF levels are similar ($p>0.05$) at the day of oocyte pick-up between women who got pregnant and who did not.	NA
(Ledee-Bataille et al., 2004)	133 women undergoing IVF-ET	ELISA	IL18	Lower IL18 in women with successful implantation and pregnancy ($p=0.02$)	IL18 level, SE=85.7% SP=35.2%
(Mikolajczyk et al., 2007)	57 infertile women (multiple causes)	ELISA	LIF	Lowered LIF levels with idiopathic infertility 7–9 days after ovulation ($p<0.01$), infertile women with LIF>2.31 pg/ml more likely to get pregnant.	LIF>2.31 pg/ml, SE=95.7% SP=81.8%
(van der Gaast et al., 2009)	34 healthy women	ELISA	LIF	LIF does not correlate with endometrial dating ($r=0.11$, $p=0.6$).	NA
(Gillott et al., 2008)	30 women undergoing IVF-ET	2D-PAGE LC/MS/MS	Fluid proteins	LRG ($p<0.05$) higher in women who became pregnant	NA

Study	Subjects	Method	Targets	Results	Proposed classifier and its performance
(Parmar et al., 2008)	5 fertile women from proliferative and 6 from MSE phase	2D-PAGE MALDI-TOF MS	Fluid proteins	3 proteins more abundant (FC≥2) in MSE phase.	NA
(Boomsma et al., 2009b)	210 women undergoing IVF-ET	Multiplex immunoassay	Cytokines	Higher and lower TNFa ($p=0.03$) and IL1b ($p=0.05$), respectively, in women with clinical pregnancy	Ratio of TNFa/ILb, AUC of 0.61
(Scotchie et al., 2009a)	10 healthy women	2D-PAGE MALDI-TOF MS/MS	Fluid proteins	82 proteins different ($p<0.001$) between ESE and MSE.	NA
(Hannan et al., 2010)	7 fertile and 8 infertile (endometrial factor suspected) women	2D-DIGE MALDI-TOF MS/MS	Fluid proteins	18 proteins different ($p<0.05$) in MSE between fertile and infertile women	NA
(Hannan et al., 2011)	8 fertile and 9 infertile women	Multiplex immunoassay	Cytokines, growth factors	VEGF lower ($p<0.05$) in women with unexplained infertility during MSE	NA
(Heng et al., 2011)	103 fertile women and women with unexplained infertility	Western blot	PCSK5	MSE levels are higher in fertile women ($p<0.01$)	NA
(Bhutada et al., 2014)	7 healthy fertile women	iTRAQ LC/MS/MS	Fluid proteins	27 proteins different between ESE and MSE ($p<0.05$)	NA
(Fitzgerald et al., 2018b)	9 fertile and 10 women with idiopathic infertility	LC/MS/MS	Fluid proteins	6 proteins different (FC≥2, $p<0.05$) between fertile and infertile women in proliferative phase	NA

2D-PAGE – 2-dimensional polyacrylamide gel-electrophoresis, DIGE – difference gel electrophoresis, AUC – area under ROC curve, ESE – early secretory phase, FC – fold change, iTRAQ – isobaric tag for relative and absolute quantitation, MSE – mid-secretory phase, NA – not available, SE – sensitivity, SP – specificity.

endometrial receptivity is still not clear (Salamonsen et al., 2013). It is also notable that recombinant LIF *per se* does not improve implantation and pregnancy rates in women with RIF, and actually led to a significantly worse pregnancy rate after embryo transfer (Brinsden et al., 2009).

MS-based proteomics studies on uterine fluid (Table 2) have generally been performed with first generation methods (*i.e.* 2D-PAGE combined with MALDI MS or MS/MS), which have poor reproducibility, low coverage and by now have been phased out in favor of more sensitive LC/MS/MS approaches. It was and somewhat still is a widespread concerning practice that many MS-based biomedical proteomics studies do not account for multiple testing and rely solely on uncorrected statistics for significance calling (Diz et al., 2011). Unfortunately, none of the proteins found in the uterine fluid proteomics studies listed in Table 2 would retain conventional significance if corrected for multiple comparisons. This is not to say that the results are inherently erroneous, but the false positive rates in those studies are higher than 5%.

2.3.5 Pathogenetic mechanisms driving the establishment, development and maintenance of endometriotic lesions

Endometriosis is a prevalent chronic gynecological disease affecting women of reproductive age with a 5–10% prevalence (Zondervan et al., 2018). This heterogeneous estrogen-dependent inflammatory condition is characterized by endometrial-like tissue proliferating in extrauterine (non-eutopic) sites and forming ectopic lesions mostly on the surfaces of peritoneal cavity organs. The ectopic lesions can be subcategorized into three phenotypes (from least to most severe): (1) superficial peritoneal lesions, (2) ovarian endometriomas and (3) deep infiltrating lesions (Chapron et al., 2019). The disease itself is categorized to four stages depending on severity scoring (Chapron et al., 2019; Vercellini et al., 2014). Endometriosis is mostly associated with reduced quality of life (moderate to severe pain, fatigue, depression) and infertility, although for some women no overt symptoms are present. No reliable diagnostic markers have been established so far (Falcone and Flyckt, 2018). Diagnostics mostly relies on patient interviews, imaging (for endometriomas and deep endometriosis) and/or laparoscopic surgery if the lesions are below visual detection limit (peritoneal lesions) for imaging technologies. Although, surgery is no longer recommended as a first-line approach due to high recurrences rates of ~40–50% and associated risks with surgery, and should be reserved only for cases where prior attempts for pregnancy have failed (Chapron et al., 2019; Guo, 2009).

The establishment of endometriotic lesions requires the transfer of endometrial cells to extrauterine sites, most commonly believed to occur through retrograde menstruation as the lesions are anatomically often found consistent with a gravity-driven peritoneal flow from the Fallopian tubes (Djokovic and Calhaz-Jorge, 2015; Vercellini et al., 2007). Nevertheless, this does not explain all cases of endometriosis, such as lesions found in rare anatomical locations (brain, lungs,

limbs, nasal cavity) and retrograde menstruation also occurs in women without endometriosis. Therefore, it has been proposed that abnormal mesenchymal stem cell (MSC) differentiation explains these occurrences better (Figueira et al., 2011). By that theory multipotent MSCs from bone marrow and/or endometrium transdifferentiate to endometrial cells found in lesions. Similar, and somewhat mutual, theory proposes lymphatic and vascular spread of uterine endometrial fragments (Jerman and Hey-Cunningham, 2015). Both of these latter transmission theories are believed to play more role for less frequent cases of endometriosis, and for case reports in literature where endometriotic lesions have, under rare circumstances, been found in men (Taguchi et al., 2012). Overall, no single theory for endometriotic lesion establishment explains all endometriosis cases and factors that affect cellular invasiveness, proliferation and survival must also be at play.

Genetic factors clearly appear to contribute to the development of endometriosis, as monozygotic twins have an estimated heritability of ~50% (Saha et al., 2015), while common SNP-based heritability is about ~25% (Lee et al., 2013). The largest genome-wide association study in endometriosis to date found strong evidence for 19 SNPs and highlighted genes involved in sex steroid hormone signaling (Sapkota et al., 2017). Depending on the severity stratification the explained risk variance was explained up to 2–5%, which implicates that majority of relevant loci are still to be discovered. Other reported loci have also implicated genes involved in Wnt signaling, cell adhesion, cell migration, angiogenesis and inflammation (Sapkota et al., 2017; Zondervan et al., 2018).

In support of genetic evidence, numerous studies have found aberrant estrogenic signaling in endometriosis. Increased aromatase activity in ectopic lesions elevates local levels of estrogens (Zeitoun and Bulun, 1999), and levels of estrogen receptors (especially ERβ relative to ERα) are increased in lesions (Bulun et al., 2012; Pellegrini et al., 2012). High ERβ-to-α is also associated with progesterone resistance and may therefore contribute to infertility seen in endometriosis (Fox et al., 2016). Amplified estrogenic signaling supports ectopic proliferation of endometriotic cells, reduces their responsiveness to apoptotic signals, increases cellular adhesiveness and also drives epithelial-mesenchymal transition (EMT) (Han et al., 2015) – latter of which may find contribution from epigenetic silencing of E-cadherin promoter (Li et al., 2017) and increased peritoneal levels of transforming growth factor-β1 (TGFB1)(Young et al., 2014). TGFB1 also appears to trigger a Warburg-like metabolic reprogramming of endometriotic cells elevating local lactate levels which further fuels cell invasion, angiogenesis and immune suppression (Hirschhaeuser et al., 2011) – all features which are relevant to endometriotic cells (Zondervan et al., 2018). Nevertheless, it is still somewhat unclear whether the cellular properties described for endometriotic tissue are inherent to endometrial cells/tissue or are rather a result of the local inflammation and fibrosis, or a mixture of both.

2.3.6 Proteomics studies on the pathogenesis of endometriosis

Proteomics studies on endometriosis pathogenesis have yielded results with limited overlap in terms of proteins reported (Table 3). Similarly to above-mentioned uterine fluid proteomics studies, majority of the investigations have been carried out with less advanced proteomics methods using relatively lax statistical criteria given the high-throughput nature of the acquired data (*i.e.* not accounting for multiple testing). Nevertheless, many of the studies have used follow-up validation and partially confirmed the high-throughput hits, mostly with immunological methods and less so with functional assays.

Results have been more congruent on general themes (either on pathway or enrichment level) that are thought to be affected in endometriosis, such as altered cellular motility/invasiveness, proliferation, involvement of cellular stress response and susceptibility to apoptosis (for references, see Table 3). Of particular note, a thorough study by Vehmas *et al* suggested activation of the TGFB1 pathway as it was found to be very significantly ($p=1.3\times10^{-21}$) implicated in ovarian endometriomas (Vehmas et al., 2014), which also agrees with results for altered (peritoneal) microenvironment described in the previous section. Nevertheless, the overall results from proteomics studies have not been very conclusive demonstrating that more rigorous studies are warranted.

Table 3. Overview of proteomic studies since 2010 on endometriosis pathogenesis.

Study	Subjects	Biological material	Methods	Targets	Results
(Chehna-Patel et al., 2010)	11 endometriosis patients, 9 controls	Eu- and ecEM	2D-PAGE MALDI MS, WB, IHC	Tissue proteins; validation for HP, ARHGDIA, TAGLN2, RAB37	11 proteins exclusive to ecEM of which 4 validated by WB, IHC (by visual observation).
(Stephens et al., 2010)	4 endometriosis patients, 4 controls; 14+18 for validation	EuEM	2D-PAGE (DIGE) MALDI MS/MS, WB, IHC	Tissue proteins; follow-up validation for VIM, RNH1, TAGLN2, PRDX6, COROA1	20 proteins differentially expressed ($p<0.05$); 3 proteins validated either with WB or IHC ($p<0.05$); VIM ↓ in endometriosis, RNH1 and PRDX6 with mixed results; CORO1A and TAGLN2 did not validate ($p>0.05$).
(Rai et al., 2010a)	24 endometriosis patients, 24 controls	EuEM	2D-PAGE MALDI MS/MS, WB	Tissue proteins; validation for PARK7, HSP27, HSP60, HSP70, GRP78, HSP90AB1, MVP and PDIA3	100/116 protein spots differentially expressed ($p<0.05$) in endometriosis in proliferative and secretory phase, respectively; most proteins found to be involved in cellular stress response, protein-folding and -turnover, immunity, energy production, signal transduction, RNA biogenesis, protein biosynthesis, and part of the nucleus or cytoskeleton; 10 proteins validated ($p<0.05$) by WB+IHC as significant in both EM phases.
(Wolfler et al., 2011)	8+8 OE and PE patients, and 8 controls	Peritoneal fluid	2D-PAGE MALDI MS, WB	Fluid proteins; validation for ITIH4, AFM, APOA4, HP	11 proteins different across groups ($FC\geq1.4$, $p<0.05$); ITIH4 (↑ in PE), AFM (↑in OE), APOA4 (↓in OE) and HP (↑ in OE and PE) validated (data was not shown).
(Hwang et al., 2013)	6+3 patients with/without endometriosis	EM cells from menstrual blood	qPCR, 2D-PAGE LC/MS/MS, IB	mRNAs of stem cell markers; EM cell proteins	Stem cell marker (Oct-4, CXCR4, SOX2, MET) mRNA levels higher ($p<0.05$) in endometriosis (in validation $p>0.05$); CRMP2, UCH-L1, MYL9 ≥3x ↓ in endometriosis (stat. sign. not reported).

Study	Subjects	Biological material	Methods	Targets	Results
(Marianowski et al., 2013)	8 endometriosis patients, 1 control	Paired eu- and ecEM (ovarian)	2D-PAGE LC/MS/MS	Tissue proteins	VIM ↑ (p=0.02) in ec- vs euEM.
(Vehmas et al., 2014)	8 endometriosis patients	Cryoslides of eu- and ecEM	LC/MS/MS, mRNA microarray, WB, IHC	Tissue proteins; validation for EMILIN1, CNN1	214 proteins differentially abundant (FC>1.5, q<0.01), 88 of those shared with microarray results (q<0.01, FC>1.5); TGB1 pathway found to be activated in ecEM of OE ($p=1.3\times10^{-21}$); EMILIN1 and CNN1 ↑in ecEM (based on visual observation).
(Weng et al., 2014)	14 endometriosis patients before/after GnRH agonist	EuEM, primary cell culture	Functional assays, 2D-PAGE (DIGE) MALDI MS, WB, IHC	Tissue proteins; validation for GRP78, PPA1, EFHA2, TGM2 (validation on same samples)	GnRH agonist induces apoptosis in EECs (p<0.05); 55 proteins differentially abundant between GnRH–/+ groups (FC≥2, p<0.05); GRP78, PPA1 and EFHA2, TGM2 validated (p<0.05) as ↓ or ↑in response to GnRH agonist, respectively; siRNA knockdown of GRP78 enhances GnRH agonist induced apoptosis in vitro (p<0.05).
(Sobel et al., 2015)	NA	Endometriotic cell line Z12	iTRAQ LC/MS/MS, WB	E2 and LXA_4 regulated proteome; CSN5 validated by WB	348/25 proteins differentially abundant across treatment groups at p<0.05/q<0.1; LXA_4 antagonizes 148 E2-regulated proteins.
(Xue et al., 2018)	3 endometriosis patients	Eu- and ecEM, ESC cell line	LC/MS/MS	Peptidome; validation for VCAM1 peptide PDFV	491 differentially abundant peptides (FC≥2, p<0.05). PDFV peptide associated with increased ESC invasiveness in vitro (p<0.05).

2D-PAGE – 2-dimensional polyacrylamide gel-electrophoresis, DIGE – difference gel electrophoresis, E2 – estradiol, eu-/ecEM – eutopic/ectopic, EM – endometrium, IHC – immunohistochemistry, iTRAQ – isobaric tag for relative and absolute quantitation, LXA_4 – lipoxin A_4, NA – not available, OE – ovarian endometriosis, PE – peritoneal endometriosis, WB – Western blot, ↑/↓ – up- or -downregulation.

2.4 Summary of the literature review

MS-based proteomics has evolved rapidly in recent times, where the number of detected proteins has increased from few hundreds since the early implementation of the technology to near the complete expressed proteome, as of date. In reproductive medical research, a large wave of proteomics studies was carried out during the emergence of the technology. However, due to the shortcomings of the initial methods, the retrieved results were often of limited coverage, quantitative accuracy and precision. Thus, a call for revisiting many of these areas in reproductive sciences is warranted to confirm initial results and widen our understanding on the role of protein dynamics in reproductive health.

The causes behind endometriosis are complex and multifactorial, and far from completely understood. Studying the role of proteins in the pathogenetic mechanisms of the disease may offer new perspectives for developing novel interventional therapeutics, and for finding diagnostic biomarkers with higher specificity and lower invasiveness. Similarly, MS proteomics is ideally suited for finding new biomarkers for monitoring endometrial receptivity. Using uterine fluid proteins would offer advantages over current biopsy-based approaches – both in terms of invasiveness and in terms of applicability in the same menstrual cycle as uterine sampling. Therefore, time is ripe for another wave of proteomics studies in reproductive medicine to complement insights from genomics and transcriptomics.

3. AIMS OF THE STUDY

The general aim of the study was to apply contemporary proteomics technologies for the study of human endometrial proteins in health and dysfunction, such as infertility and endometriosis. The specific aims were:

1. To shed light to the pathogenetic processes occurring in endometriotic lesions by characterizing the proteomes of ectopic and eutopic stromal cells.
2. Perform a discovery proteomics study to find potential novel and specific biomarkers from immunodepleted blood plasma for the non-invasive diagnostics of endometriosis.
3. Characterize the proteomic profile of secretory phase uterine fluid and how it changes in transition from a pre-receptive state to a receptive one.
4. Study tissue expression and cellular localization of selected uterine fluid proteins indicative of endometrial receptivity.
5. Determine if minimally invasively obtainable uterine fluid is suitable for monitoring of endometrial receptivity, and, whether it is altered in women with recurrent implantation failure of unknown origin.

4. MATERIALS AND METHODS

4.1 Ethics statement and study participants

All four independent studies within this thesis were carried out in accordance with approved guidelines. The studies were approved by the Ethics Committee of University of Tartu, Karolinska University Hospital and by the Institutional Review Board of University of California, San Francisco (UCSF).

The subjects of the studies included healthy controls, endometriosis patients and women with RIF. Study subjects were recruited through advertisements, hospitals or IVF clinics. Archived endometrial biopsy samples were obtained from a biobank. Informed written consent was obtained from all participants. In addition, participants were asked to fill out medical questionnaires to obtain thorough information regarding general and reproductive health characteristics, including menstrual cycle anamnesis, use of medications, presence of systemic diseases and other health conditions.

Volunteers comprising control groups in studies I (endometrial biopsies), II, III (uterine lavages) and IV (endometrial biopsies and uterine lavages) were self-reported healthy women recruited through advertisement or through IVF clinics undergoing first attempt IVF for male-factor infertility or had tubal factor infertility (TFI). All control women had regular menstrual cycles of 28±5 days without any evidence of endometriosis, polycystic ovary syndrome (PCOS), primary ovarian insufficiency (POI) or uterine abnormalities (*e.g.* fibroids, adenomyosis). Women in control groups in studies I, II (discovery cohort), III (uterine lavages) and IV were with proven parity. Plasma control group in study I included all women with endometriosis-like symptoms (*e.g.* pelvic pain, infertility) but were laparoscopically determined as not having endometriosis. Control group in study III comprised women undergoing gynecologic surgery for fibroid management or tubal ligation. All control women in the studies were not pregnant nor taking contraceptive medications for the last 3 months.

The diagnosis of endometriosis for patients in studies I and III was determined by visual observation of lesions during laparoscopic surgery and confirmed by histology. Endometriosis staging was carried out using the American Society for Reproductive Medicine revised classification system (1997). The RIF cohort in study II comprised women undergoing repeated IVF [4.5±2.0 (range: 3–10) failed previous cycles] due to male-factor infertility or TFI, and were without endometriosis, PCOS, POI or uterine abnormalities. All patients in the studies were not pregnant or taking contraceptive medications for the last 3 months. The main characteristics of all the cohorts participating in the studies are summarized in Table 4.

Table 4. Clinical characteristics of different study cohorts.

Study	Biological material analyzed	Study group	N	Mean age ± SD (range), yrs	Mean BMI ± SD (range), kg/m^2	Cycle phase
I	Eutopic and ectopic (peritoneal) endometrial stromal cells	Endometriosis patients (Stage I, n=4; II, n=1; III, n=7; IV, n=1)	13	31.4±5.0 (23–39)	22.8±3.8 (16.1–29.1)	Secretory
	Eutopic endometrial stromal cells	Healthy fertile women	6	30.2±3.9 (25–34)	21.7±2.1 (18.9–23.4)	Secretory
	Blood plasma	Endometriosis patients (Stage I–II n=60; III–IV n=59)	119	31.1±4.3 (21–40)	22.3±3.3 (16.1–33.0)	Follicular and luteal
		Laparoscopically confirmed non-endometriosis women	53	31.5±3.9 (22–39)	23.3±3.8 (18.8–35.7)	Follicular and luteal
II	Uterine fluid	Healthy, fertile women	17 (n=6 with proven parity)	29.9±3.5 (22–36)	23.2±3.3 (18.9–31.1)	Secretory (ESE and MSE)
		RIF (TFI n=13, male-side infertility n=16)	29	35.7±3.7 (26–42)	23.6±4.0 (19.0–38.9)	Secretory (MSE)
III	Endometrial biopsy	Control	20	36.7±6.0 (23–49)	NA	Proliferative and secretory
	Uterine fluid	Healthy fertile women	6	31.0±1.5 (29–33)	NA	Secretory (ESE and MSE)
IV	Endometrial biopsy	Healthy fertile women	36	(22–37)	NA	Proliferative and secretory
	Uterine fluid	Healthy fertile women	6	31.0±1.5 (29–33)	NA	Secretory (ESE and MSE)

BMI – body mass index, ESE – early secretory phase, MSE – mid-secretory phase, NA – not available, PCOS – polycystic ovary syndrome, POI – premature ovarian insufficiency, SD – standard deviation, TFI – tubal factor infertility, RIF – recurrent implantation failure, follicular phase – menstrual cycle day 1–14, luteal phase – menstrual cycle day 15–28.4.2 Clinical collection of samples

4.2 Clinical collection of samples

Endometrial and/or endometriotic peritoneal lesion biopsies and uterine lavages were collected by skilled clinicians in the Tartu University Hospital, Karolinska University Hospital or obtained from the UCSF National Institutes of Health Human Endometrial Tissue and DNA Bank.

Endometriotic lesion biopsies were collected under general anesthesia during laparoscopic surgery and endometrial biopsies were simultaneously obtained using an endometrial suction catheter (Pipelle, Laboratoire CCD). Endometrial biopsies from controls were collected under local anesthesia. After biopsy extraction, samples were immediately placed in an ice-cold 1:1 mixture of Dulbecco's Modified Eagle's Medium (DMEM) and Ham's F-12 for transport to the laboratory.

The procedure for collecting uterine fluid was done by flushing the uterus with 0.5 ml of saline for approximately 30 s followed by aspiration of the fluid. Lavages were performed with an intrauterine insemination catheter (Cooper Surgical) inserted through the cervical canal into the uterine cavity, while avoiding touching of the uterine fundus. Samples were then cleared of cells or tissue debris by centrifuging at 400 g for 5 min at 4 °C, the supernatants were carefully transferred to new tubes and stored at –80 °C until further processing.

Blood plasma samples were collected in the Tartu University Hospital. Venous blood samples were collected to EDTA tubes, centrifuged at 1,600 g for 10 min at 4 °C, the top plasma layer was transferred to a new tube and centrifuged at 16,000 g for 10 min at 4 °C. The supernatant was then frozen at –80 °C for storage until further processing.

The beginning of the endometrial secretory phase was determined by monitoring for the surge in the urinary concentration of luteinizing hormone (LH+0) with a urinary ovulation prediction test. Early secretory or pre-receptive phase (ESE) samples were collected 1–3 days after the LH+0, while the mid-secretory or receptive phase (MSE) samples were collected 6–9 days after LH+0. Additionally, MSE phase histology samples for endometrial dating according to the Noyes *et al* criteria (Noyes et al., 1950) were taken from control women in studies I, II (discovery cohort), III and IV. MSE biopsies were taken after collecting the uterine lavages.

4.3 Endometrial cell purification and cell culture (Study I and II)

Biopsies in ice-cold 1:1 DMEM and Ham's F-12 were washed with 7 ml of fresh medium to remove tissue debris and blood cells. Tissue was then dissociated in 5 ml DMEM without phenol red containing 0.5% collagenase (Sigma-Aldrich) with a shaking rotator at 110 rpm 37 °C up to 1 h or until collagenase digestion was complete. To remove undigested tissue pieces dispersed cells were filtered through a 50 µm nylon mesh. Isolation of stromal and glandular cells was

performed as described previously (Cervello et al., 2010). Briefly, the cells resuspended in 10 ml of culture medium in a 15 ml tube were placed in an upright position for 10 min for the sedimentation of epithelial glands. The top 8 ml of medium containing free-floating stromal cells was aspirated from the sedimented epithelial cells in the bottom. The process was repeated three times. Final purification was carried out by selective adherence of stromal cells to culture dishes for 30 min at 37 °C in 5% CO_2. Non-adhering epithelial cells were aspirated by washing the culture dishes twice with 5 ml of culture medium. Epithelial glandular cell samples were spun down and stored at –80 °C until further sample preparation.

Purified ESCs were cultured 2–5 passages in DMEM/F12, 10% fetal bovine serum (FBS) with 100 U/ml penicillin, 100 µg/ml streptomycin, 0.25 µg/ml amphotericin B at 37 °C and under 5% CO_2. Confluent cells were dissociated with 0.25% trypsin-EDTA (Gibco), sedimented by centrifugation at 200 g for 6 min and washed twice with 2 ml PBS each. Cell pellets were kept at –80 °C until further sample preparation and mixing with the SILAC spike-in standard.

To prepare the SILAC standard ESCs from a healthy endometrium and Ishikawa cancer cell-line were separately cultured for 6 passages in DMEM in the presence of 0.266 mM heavy ($^{13}C_6{}^{15}N_2$) lysine (Lys8) and 0.133 mM heavy ($^{13}C_6{}^{15}N_4$) arginine (Arg10) (Cambridge Isotope Laboratories) and 10% dialyzed FBS (Thermo Fisher Scientific). 200 mg/L light proline was also added to the labelling medium to suppress arginine-to-proline inter-conversion (Bendall et al., 2008).

4.4 Preparation of cell samples for non-targeted LC/MS/MS (Study I)

Cell pellets were processed by the enhanced filter-assisted sample preparation protocol (Erde et al., 2014) with modifications. Briefly, cell pellets were suspended in 10 volumes of 4% sodium dodecyl sulfate (SDS), 100 mM Tris-HCl pH 7.5, 10 mM dithiothreitol (DTT) and incubated at 95 °C for 5 min. Solutions were probe sonicated (Bandelin) with 20x 1 sec pulses at 50% power. Protein concentrations were measured in 8 M urea, 100 mM Tris-HCl pH 7.5 using tryptophan fluorescence (excitation/emission wavelengths of 295/350 nm, respectively). Samples were then spiked 1:1 with the SILAC standard. The standard consisted of 2:1 mix of labelled ESCs from a healthy donor and labelled Ishikawa cells. Samples were on-filter (30 kDa molecular weight cut-off; Millipore), alkylated with 50 mM iodoacetamide (IAA) and digested overnight with 1:50 dimethylated porcine trypsin (Sigma-Aldrich) in the presence of 1.0% sodium deoxycholate. The detergent was removed by acid precipitation and extraction with ethyl acetate. The digested proteins were pre-fractionated into six fractions using strong cation exchange (SCX) (Figure 7, “Cells”) and desalted with in-house made C18 (3M Empore) solid phase extraction StageTips (Rappsilber et al., 2007). Final LC/MS/MS samples were reconstituted in 0.5% trifluoroacetic acid (TFA).

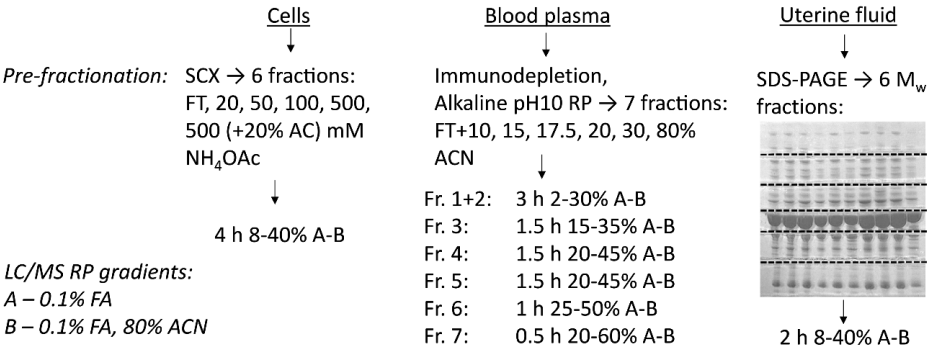


Figure 7. Peptide offline pre-fractionation and online chromatography of different proteome samples. Abbreviations: ACN – acetonitrile, FA – formic acid, FT – flow-through, OAc – acetate, AC – acetone, RP – reversed phase, SCX – strong cation exchange, SDS-PAGE – sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

4.5 Preparation of blood plasma samples for non-targeted LC/MS/MS (Study I)

Frozen plasma was thawed on ice and pooled based on diagnosis, endometriosis stage and menstrual cycle phase as outlined in Figure 8. Altogether 36 pools were created, each pool having plasma from 4–5 individuals. The processing order of pools was randomized to neutralize potential systematic technical biases.

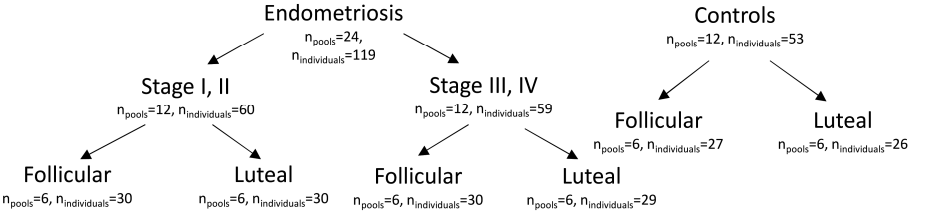


Figure 8. Experimental design for blood plasma pooling.

The pools were immunodepleted for the 14 most abundant proteins (albumin, IgG, antitrypsin, IgA, transferrin, haptoglobin, fibrinogen, alpha2-macroglobulin, alpha1-acid glycoprotein, IgM, apolipoprotein AI, apolipoprotein AII, complement C3, transthyretin) found in human blood plasma using the Multiple Affinity Removal Column Human 14 spin-cartridge kit (MARS-14, Agilent) according to the manufacturer's instructions. Briefly, 8 µl of plasma were combined with 192 µl of a mild urea-based buffer and filtered through a 0.22 µm syringe filter. The immunoaffinity spin-cartridge was then pre-equilibrated with the carrier buffer and the sample loaded on to the cartridge, centrifuged for 1 min at 100 g and the flow-through (FT) collected. The spin cartridge was then incubated for 5 min at room temperature and washed twice with 400 µl of the carrier buffer by

centrifugation at 100 g for 2.5 min. The bound proteins were eluted by washing with a concentrated urea buffer and the spin-cartridge was re-equilibrated for another round of depletion. Both washes and the FT were combined ('the depleted plasma') and precipitated with 2:1:3 (v/v/v) methanol:chloroform:water precipitation, the precipitated protein pellets were washed with –20 °C chilled methanol and air dried. The pellets were suspended in 7 M urea, 2 M thiourea, 100 mM ammonium bicarbonate (ABC) solution (7/2 urea:thiourea buffer). After reduction and alkylation of cysteines with 5 mM DTT and 20 mM chloroacetamide (CAA), respectively, for 1 h at room temperature in the dark, the samples were digested 4 h in 1:50 (enzyme:protein) ratio using *Achromobacter lyticus* Lys-C (Wako Pure Chemical Industries). Solutions were diluted five times with 100 mM ABC and further digested overnight at room temperature with 1:50 dimethylated porcine trypsin (Sigma Aldrich). Digested samples were then fractionated into seven fractions using alkaline (pH 10) reversed phase C18 StageTips (Figure 7, "Blood plasma"). Prior to injection to LC/MS/MS, samples were acidified with TFA to pH ~2.

4.6 Preparation of uterine fluid samples for non-targeted LC/MS/MS (Studies I, III and IV)

Uterine fluids were thawed on ice, measured for protein concentration using the Micro BCA kit (Thermo Fisher Scientific) and prepared for XCell SureLock Mini SDS-PAGE (Invitrogen) system according to the manufacturer's instructions. Samples were electrophoresed using the NuPAGE 4–12% Bis-Tris gradient gels (Invitrogen). Gels were stained with SimplyBlue SafeStain (Invitrogen) and each lane was sliced into six fractions (Figure 7, "Uterine fluid"). Each fraction was further cut into ~1 mm^3 pieces to increase contact with downstream processing solutions. The gel material was destained with vortexing in 1:1 ACN:100 mM ABC, reduced with 10 mM DTT at 56 °C and alkylated with 50 mM IAA in the dark. Proteolysis was carried out with 10 ng/µl of dimethylated porcine trypsin (Sigma Aldrich) in 100 mM ABC at 37 C overnight. Peptides were extracted from the gel matrix using bath sonication, 30 min vortexing in 2 volumes of 1:2 5% FA:ACN. Organic solvents were evaporated in a vacuum-centrifuge and peptides desalted with C18 tips. Samples were reconstituted in 0.5% TFA.

4.7 Non-targeted full proteome LC/MS/MS and raw data analyses (Studies I–IV)

Samples were injected to an Ultimate 3000 RSLCnano system (Dionex) equipped with a 0.3 × 5 mm trap-column (5 µm C18 particles, Dionex) and an in-house packed (3 µm C18 particles, Dr Maisch) analytical 50 cm × 75 µm emitter-column

(New Objective). Detailed pre-fractionation strategies and chromatographic conditions for different full proteomes are outlined in Figure 8. Overall, peptides were eluted at flow rates of 200–250 nl/min with continuous A to B gradients (buffer A: 0.1% formic acid (FA), buffer B: 80% acetonitrile (ACN), 0.1% FA,) to a quadrupole-orbitrap Q Exactive Plus (Thermo Fisher Scientific) mass spectrometer (MS) using a nano-electrospray source (spray voltages of 2.4–2.6 kV). The MS was operated in positive polarity with a top-N (top 5, 10 and 15 for uterine fluid, blood plasma and cellular proteomes, respectively) DDA strategies. One measurement cycle consisted of a 350–1400 m/z MS1 scan at a resolution setting of R=70,000 at 200 m/z which was followed by MS/MS scans of the 5/10/15 most intense ions (z: +2 to +6) at R=17,500. Normalized collision energy using higher-energy collisional dissociation (HCD) was set to NCE=26, isolation width was 1.5 m/z. MS and MS/MS ion target values were 3e6 and 5e4 ions, respectively, using 60 ms injection times. Dynamic exclusion varied from 20–70 s depending on total chromatographic time.

MS raw data were processed with the MaxQuant 1.4.0.8 software package (Cox and Mann, 2008). For SILAC samples, Lys8 and Arg10 were defined as the heavy channel amino acids. Methionine oxidation, asparagine/glutamine deamidation and protein N-terminal acetylation were defined as variable modifications, while cysteine carbamidomethylation was set as a fixed modification. Peptide search was performed against *in silico* trypsin digested (C-terminal cleavage after lysine/arginine without proline restriction) UniProt (www.uniprot.org) *Homo sapiens* reference proteome database. First and main search MS mass tolerances were ±20 and ±4.5 ppm, respectively. MS/MS mass accuracy tolerance was ±20 ppm. Protein identifications were reported if ≥1 razor or unique peptides of ≥7 amino acids were identified. Transfer of peptide identifications (match between runs) based on accurate MS1 mass and RT was allowed. For SILAC samples, protein quantification was reported if ≥2 H/L ratio measurements with ≥3 points across a chromatographic peak were available. For label-free samples, protein quantification was reported if ≥1 peptide was quantified with ≥3 points. Label-free protein intensities were normalized using the MaxLFQ algorithm (Cox et al., 2014). Signal integration of missing SILAC label channels (re-quantification) was also enabled. Peptide-spectrum match and protein false discovery rate (FDR) were kept ≤1% using a target-decoy approach. All other parameters were default.

4.8 RNA extraction and quantitative real-time PCR (Studies I, III and IV)

RNA was extracted from cells and tissue biopsies according to the manufacturer's instructions using RNeasy MinElute Cleanup kit (Qiagen) (Study I, III) or Arcturus PicoPure Frozen RNA Isolation kit (Thermo Fisher Scientific) (Study IV). DNase treated samples were converted to cDNA with RevertAid First Strand cDNA Synthesis Kit (Thermo-Fisher Scientific) (Study I) or iScript cDNA Synthesis kit

(Bio-Rad Laboratories) (Study III) or NUGEN Ovation Pico WTA System (NuGEN Technologies) (Study IV). Quantitative real-time polymerase chain reaction (qRT-PCR) was performed with 5×HOT FIREPol EvaGreen qPCR Mix Plus (ROX) master mix (Solis BioDyne) in 7500 Fast Real-Time PCR System (Applied Biosystems) (Study I, III) or with TaqMan Universal PCR Master Mix (Applied Biosystems) in a StepOne Plus instrument (Applied Biosystems) (Study IV). HPRT1 (Study I), RPL18 (Study III) and 18S ribosomal RNA (Study IV) were used as reference genes. The $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001) was used for calculating the relative expression and to determine mRNA expression fold changes (FC) along with paired (Study I) or non-paired (Study III, IV) t-test with significance threshold set at $p<0.05$. Primer sequences are available in the publications associated with the respective studies.

4.9 Respirometry (Study I)

Cellular routine respiration measurements were carried out with high-resolution respirometry (Oroboros Instruments) using a Clark electrode at 37 °C. Approximately 10^6 cells of paired endometriotic eutopic and ectopic ESC cultures were transferred into respiratory chambers of the oxygraph. After steady-state respiratory flux (V_r) was established, the ATP synthase was inhibited with 2 µg/ml oligomycin (V_{Oly}), followed by uncoupling of the oxidative phosphorylation by stepwise titration of carbonyl cyanide p-trifluoromethoxyphenylhydrazone (FCCP) up to optimum concentrations in the range of 2–10 µM ($V_{FCCPmax}$). Finally, respiration was inhibited and measured in the presence of rotenone (V_{Rot}, complex I inhibition) at 2.5 µM, and then, antimycin A (V_{AntA}, complex III inhibition) at 2.5 µM. Oxygen consumption slopes were calculated with DatLab 4.0 (Oroboros).

4.10 Uterine fluid sample preparation for targeted LC/MS/MS (Study II)

Proteins were directly precipitated from uterine fluids with 10% trichloroacetic acid (TCA) overnight at 4 °C. The precipitated protein pellets were washed with –20 °C chilled acetone and air dried. Protein concentrations were determined with the Micro-BCA assay (Thermo Fisher Scientific). 15 µg of uterine fluid proteins were then suspended in the 7 M urea/2 M thiourea buffer in 100 mM ABC, reduced with 5 mM DTT and alkylated with 20 mM CAA. Pre-digestion with 1:50 Lys-C was carried out for 4 h at room temperature, followed by overnight digestion with 1:50 trypsin. Samples were desalted with C18 SPE tips and reconstituted in 0.5% TFA.

4.11 Targeted LC/MS/MS and raw data analysis (Study II)

Samples were injected to an Ultimate 3000 RSLCnano system with a configuration as described in paragraph 4.9 above. Peptides were eluted at 250 nl/min with a 90 min A to B 10–45% gradient to a Q Exactive Plus MS/MS using a nano-electrospray source (positive mode, spray voltage of 2.6 kV). The MS was operated in a scheduled PRM mode by isolating and fragmenting only selected peptides from proteins selected for targeted validation. Scheduled MS/MS was performed within ±3 min windows of their predicted RTs by using the indexed retention time (iRT) method (Escher et al., 2012). Briefly, iRT indices were determined for all peptides from the validation set in method development runs using a spiked-in iRT calibration mix (Biognosys). MS/MS isolation window was 1.0 m/z with an ion target value and fill time of 2e5 ions and 160 ms, respectively. HCD NCE was set to 26. MS raw files were analyzed with the Skyline software (MacLean et al., 2010). Spectral library was created from the previously measured uterine fluid full proteome data. Peptides with the highest intensities in the library were preferred for the targeted analysis. No restrictions on amino acid composition were enforced, as this was found to exclude many peptides with strong MS response factors. For quantitative analysis only y-ion fragments (from ion 3, y_3) with +1 and +2 charges states were allowed. After automated extracted ion chromatogram integrations, all integrations were manually inspected for correct peak picking. Ion traces with strong interference and erroneously picked peaks (*i.e.* with mass errors > ±20 ppm, lack of fragment chromatographic co-elution) were discarded. Integrations were then exported and processed with an in-house written R script. Peptide AUCs were summed into protein AUCs, normalized using EEF1A1 AUCs and $\log_2$-transformed. Missing values were imputed using MaxQuant's Perseus module as described in paragraph 4.9 above. Imputation was not performed for proteins in paired samples where both ESE and MSE values were absent.

4.12 Immunohistochemistry (Study III and IV)

Paraffin-embedded endometrial slides of 4–5 µm were deparaffinized, washed and processed for antigen retrieval with either submerging in citrate buffer at 90 °C for 10 min or using a 2100-retriever autoclave (Biocare). Endogenous peroxidase was inactivated with 3% H_2O_2 in methanol and the slides were incubated with 1:150 rabbit anti-human polyclonal anti-stanniocalcin 1 (STC1) (Santa Cruz) or 1:500 rabbit anti-human polyclonal ectonucleotide pyrophosphatase/phosphodiesterase family member 3 (ENPP3) (Sigma Aldrich) primary antibodies at 4 °C overnight. STC1 detection was performed with 1:300 goat anti-rabbit secondary antibody (Vector Laboratories) and ENPP3 with Rabbit/Mouse HRP polymer kit MACH 3 (Biocare Medical). STC1 slides were developed with the Vectastain Elite ABC immunoperoxidase detection and DAB kits (Vector Laboratories), ENPP3 slides were developed with the Betazoid DAB Chromogen

kit (Biocare Medical). After counterstaining with hematoxylin, slides were mounted and analyzed with a microscope by two blinded observers. ENPP3 staining intensity was quantified with the Immunoreactive Score (IRS) and STC1 was quantified by a five-grade scale (0 – no staining, 1 – few stained cells, 2 – faint, 3 – moderate, 4 – strong staining). Kruskal-Wallis test (Study III) and Mann–Whitney U test (Study IV) were used for significance testing at $p<0.05$.

4.13 Bioinformatics and statistical analyses of proteomics data

For the SILAC experiment (Study I), statistical analyses were carried out using the MaxQuant Perseus software and Microsoft Excel's Real Statistics Resource Pack. To account for any SILAC mixing errors the median peptide log H/L ratio was shifted to zero based on the assumption that most proteins across conditions do not change. Ratios were then inversed, $\log_2$-transformed and proteins were filtered to have at least three measurements per group. Measured ratios were checked for conformity with the normal distribution. One-way analysis of variance (ANOVA) with multiple testing correction using the Benjamini & Hochberg's FDR procedure ($FDR<0.05$) was used to detect significantly changing proteins. Post-hoc comparisons and significance ($p<0.05$) were determined with the Tukey's honestly significant difference (HSD) test.

Term enrichment analysis for the SILAC data was carried out with the Database for Annotation, Visualization and Integrated Discovery (DAVID) bioinformatics resources (Huang da et al., 2009) using the significantly up- or down-regulated UniProt (www.uniprot.org) identifiers as the search input. Human expressed genome was set as the enrichment background and EASE score of 0.1 was set as the enrichment cut-off value. DAVID EASE score is a more conservatively modified p-value of the Fisher Exact test. For general data mining purposes we used an enrichment q-value of ≤ 0.1 for Kyoto Encyclopedia of Genes and Genomes (KEGG) Pathway and Gene Ontology Biological process (GOBP) terms.

For label-free proteomics data, MaxQuant label-free protein intensities (LFQ) were imported to the Perseus software environment. The LFQ values were $\log_2$-transformed and proteins were filtered to have ≥50% valid values in sample groups. Missing values were then imputed or randomly drawn from a distribution created by down-shifting and compressing the measured intensity distributions by 1.8 and to 0.3 standard deviation units, respectively. Thereby simulating intensities on the threshold of MS detection and enabling statistical analysis for proteins where their abundance is negligible in certain conditions, but high in others. Statistical difference was tested with either paired or non-paired samples t-test as appropriate. All p-values were corrected for multiple testing with the Storey's q-value method (Storey and Tibshirani, 2003). Proteins with a q-value <0.05 were considered significantly different. The targeted MS validation data in

Study II was analyzed either with paired (for paired ESE and MSE samples) or independent (for MSE and RIF MSE samples) t-test, proteins were considered validated if $p<0.05$.

Principal component analysis (PCA) was used to study the ability of proteins to distinguish different groups of samples in the targeted MS validation dataset (Study II) and was conducted with the R packages FactoMineR and factoextra (Le et al., 2008). Term enrichment analyses in Study II were conducted with the FunRich software using UniProt 'Cellular localization' and Gene Ontology 'Biological process' terms (Pathan et al., 2015). Gene enrichment was estimated with the Fisher's exact test against the expressed human genome and adjusted for multiple testing with the Benjamini-Hochberg's FDR method.

To estimate the classification accuracy (sensitivity and specificity) of different protein sets in Study II we used Random Forest machine learning with the R package randomForest (Breiman, 2001). Different 3- and 4-protein panels from the 21 proteins that were validated to be implicated in endometrial receptivity and showed displacement in RIF (AOC1, CD55, CRISP3, CTSB, DPP4, ELANE, ENPP3, GRN, LCN2, MMP26, MPO, MSLN, MVP, NNMT, PGR, RNASET2, SDCBP2, SLC26A2, SLC34A2, STC1 and TCN1 were combinatorically generated and their classification performance was evaluated using the Random Forest supervised machine learning approach. Paired ESE and MSE samples were essentially treated as independent samples (*i.e.* both representing random samples of the studied phases), as the requirement for paired samples in clinical practice is inconvenient, and receptivity status is preferably determined based on a single MSE sample collected at a post-LH surge day of 6–9. The Random Forest sensitivity and specificity estimates are then similar to the cross-validation estimates and reduce the over estimation of classification accuracy.

5. RESULTS

5.1 Profiling of proteomes of endometriotic cells (Study I)

5.1.1 Proteomes of primary stromal cells from endometriotic lesions differ compared to cells from the endometrium (Study I)

Using a spike-in super-SILAC standard and fractionation based quantitative proteomics workflow (Figure 9) we were able to identify and quantify ~6,900 and ~4,000 proteins across samples, respectively, from primary stromal cell samples obtained from peritoneal endometriotic lesions and endometrium of endometriosis patients and healthy controls. For the standard, we used a mixture of heavy labelled (incorporation ~98%) secretory phase ESCs of healthy women and EECs of Ishikawa endometrial cancer cell line (Nishida et al., 1985).

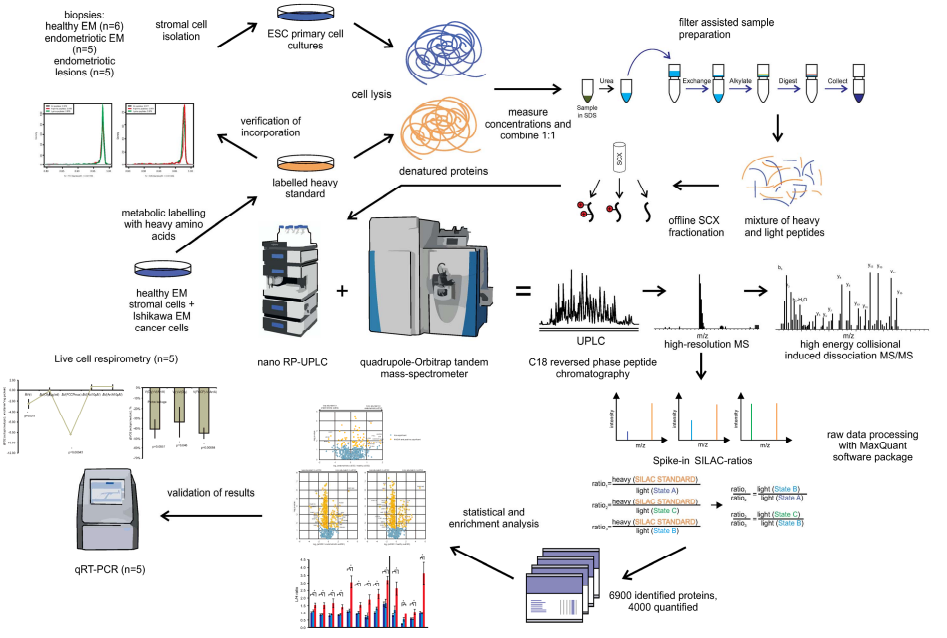


Figure 9. Experimental scheme for super-SILAC internal standard-based quantitative proteomics workflow for investigating primary stromal cells from endometriosis patients and healthy controls.

It was evident from the comparative results that primary ectopic ESCs (ecESC) are significantly more different from their eutopic counterparts (euESCs) than endometriosis euESCs versus control euESCs (Figure 10). In fact, 1,492 and 1,437 proteins were differentially expressed (ANOVA $q<0.05$, post-hoc $p<0.05$) when comparing ecESCs to endometriosis euESCs and control euESCs, respectively, while control and endometriosis euESCs had only 110 proteins differentially expressed. This magnitude of differences was also reflected in effect sizes with

ecESCs having many fold higher significant changes over euESCs. Also, in hierarchical clustering ecESC clearly clustered separately, while distinction between endometriosis and control euESCs was less pronounced (Figure 11). Proteins with strong effect sizes (≥4-fold difference) in ecESCs over euESC comparisons (listed in Table 5) were subjected to literature search to find their potential relevance for endometriosis.

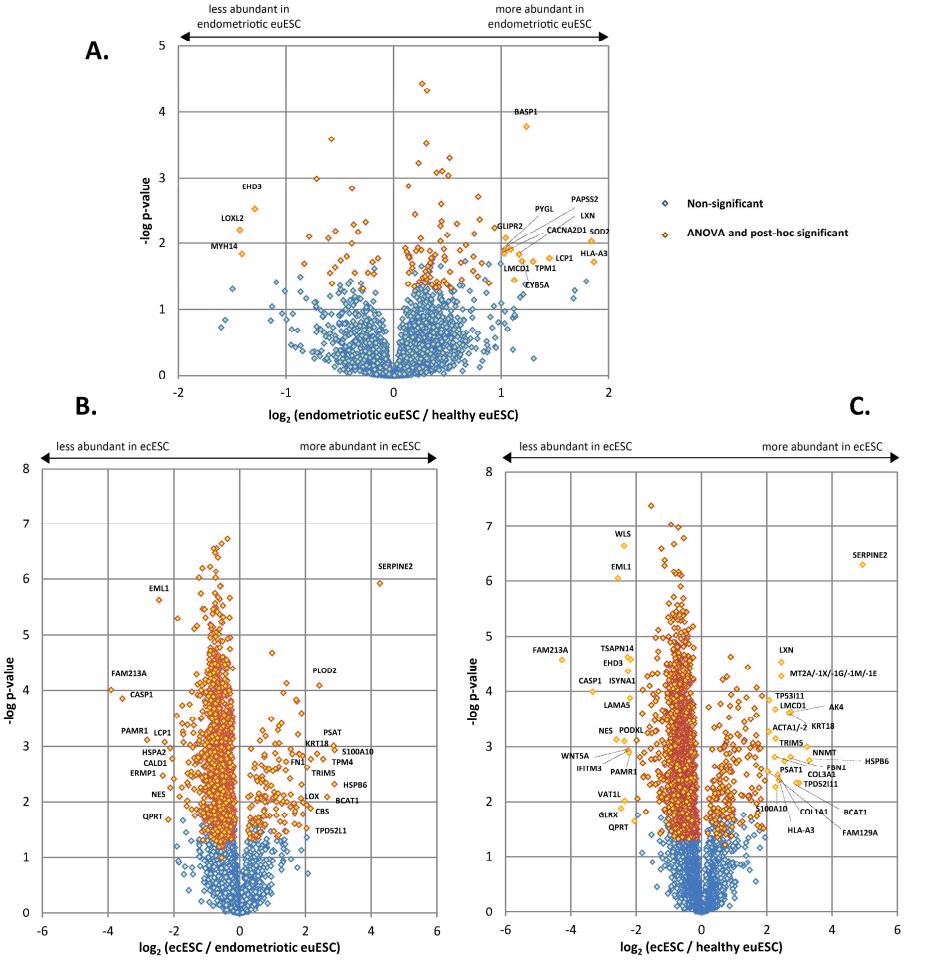


Figure 10. Volcano plots of different ESC comparisons indicate stark contrasts between primary cells from ecESC vs euESC. **A.** Endometriosis euESCs compared to control euESCs. **B.** ecESCs compared to endometriotic euESCs. **C.** ecESCs compared to control euESCs. Data were analyzed with one-way ANOVA (Benjamini & Hochberg FDR ≤0.05) and significantly changing proteins pair-wise tested with Tukey-Kramer's post-hoc test ($p \leq 0.05$ considered as a significant difference). x- and y-axis values have been obtained by taking $\log_2$ and $\log_{10}$ of the ratio of mean L/H ratios and post-hoc test p-values of indicated ESC sample pairs, respectively. Significantly changing proteins have been indicated in yellow.

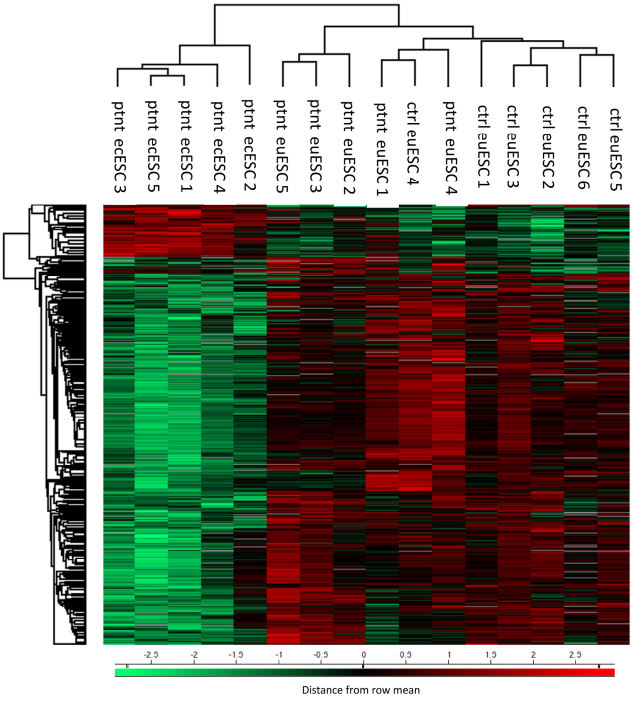


Figure 11. Hierarchical clustering of ecESC and euESC samples based on ANOVA significant proteins distinctly group the ecESC samples apart from euESCs. L/H ratios were z-score normalized and column/row clustering was based on Pearson correlation and Euclidean distance, respectively. Abbreviations: ptnt ecESC – ESCs from peritoneal lesions of endometriosis patients, ptnt euESC – ESCs from endometrium of endometriosis patients, ctrl euESC – ESCs from endometrium of healthy controls.

Table 5. Proteins with over fourfold difference between ecESC vs control euESC or ecESCs vs endometriotic euESCs.

Protein name	**Gene**	**Direct or potential relevance to endometriosis**	**Protein FC** (ecESC vs control euESC)	**p-value** (ecESC vs control euESC)	**Protein FC** (ecESC vs endometriotic euESC)	**p-value** (ecESC vs endometri-otic euESC)
Glia-derived nexin, Isoform 2/3	SERPINE2	Enhancement of cellular invasiveness (Yang et al., 2018).	+30.5	5.0E-07	+19.2	1.2E-06
Heat shock protein beta-6	HSPB6	Response to stress, angiogenesis (Zhang et al., 2012).	+9.9	1.8E-03	+7.4	4.8E-03
Nicotinamide N-methyltransferase	NNMT	Induction of cellular invasiveness(Tang et al., 2011).	+9.4	1.0E-03	+3.0	1.1E-01
Tumor protein D53	TPD52L1	Inhibition of MAP3K4 induced apoptosis, upregulated in neoplasms (Pekow et al., 2013).	+7.9	4.6E-03	+4.1	3.0E-02
Branched-chain-amino-acid aminotransferase, cytosolic	BCAT1	Increased cell viability, promotion of proliferation and migration (Zeng et al., 2019).	+7.5	4.6E-03	+6.3	8.1E-03
Keratin, type I cytoskeletal 18	KRT18	Negative regulation of apoptosis (Oshima, 2002).	+6.7	2.3E-04	+5.1	1.4E-03
Fibrillin-1	FBN1	ECM reorganization, cellular adhesion (Stumm and Zorn, 2007).	+6.6	1.5E-03	+3.2	2.5E-02
GTP:AMP phosphotransferase AK4, mitochondrial	AK4	Regulation of energy charge; enhancement of cellular invasiveness (Jan et al., 2012).	+6.4	2.5E-04	+3.1	1.1E-02
Phosphoserine aminotransferase	PSAT1	Enhancement of cellular proliferation (Gao et al., 2017).	+5.8	1.8E-03	+7.2	9.8E-04
Latexin	LXN	Participation in inflammatory responses (Aagaard et al., 2005).	+5.5	2.9E-05	+2.4	1.1E-02
Metallothionein-2/1X/1G/1M/1E	MT2A/-1X/-1G/-1M/-1E	Enhancement of cellular invasiveness (Kim et al., 2011).	+5.5	5.3E-05	+3.5	1.4E-03

Protein name	**Gene**	**Direct or potential relevance to endometriosis**	**Protein FC** (ecESC vs control euESC)	**p-value** (ecESC vs control euESC)	**Protein FC** (ecESC vs endometriotic euESC)	**p-value** (ecESC vs endometri-otic euESC)
HLA class I histocompatibility antigen, A-3 alpha chain	HLA-A3	Inhibition of NK-mediated killing of endometrial cells in culture (Semino et al., 1995).	+5.2	2.3E-03	+1.4	6.9E-01
Collagen alpha-1(I) chain	COL1A1	Fibrotic characteristics of endometriotic lesions (Zheng et al., 2016)	+5.1	3.2E-03	+3.8	1.7E-02
Tripartite motif-containing protein 5	TRIM5	NA	+4.8	7.2E-04	+4.1	2.4E-03
Protein S100-A10	S100A10	Promotion of cellular migration (Saiki and Horii, 2019).	+4.8	5.4E-03	+7.4	1.2E-03
LIM and cysteine-rich domains protein 1	LMCD1	Promotion of cellular migration, tumour metastasis (Chang et al., 2012).	+4.8	2.1E-04	+2.2	4.2E-02
Collagen alpha-1(III) chain	COL3A1	Fibrotic characteristics of endometriotic lesions; upregulated in endometriotic lesions (Hever et al., 2007).	+4.7	1.5E-03	+3.2	1.7E-02
Tumor protein p53-inducible protein 11	TP53I11	Suppression of HIF1A (Xiao et al., 2019).	+4.2	1.4E-04	+2.5	7.6E-03
Actin, aortic smooth muscle	ACTA2; ACTA1	NA	+4.2	5.4E-04	+3.8	1.5E-03
Protein Niban 1	FAM129A	Promotion of tumour growth (Pallmann et al., 2019).	+4.1	2.8E-03	+3.7	4.9E-03
Tropomyosin alpha-4 chain, Isoform 2	TPM4	Cellular contraction, potential autoantigen in endometriosis (Gajbhiye et al., 2012).	+3.6	2.8E-02	+5.8	1.7E-03
Cystathionine beta-synthase	CBS	Increased cellular chemoresistance (Santos et al., 2019).	+3.5	3.0E-02	+4.5	1.3E-02
Fibronectin	FN1	Cellular adhesion, resistance to apoptosis, upregulated in endometriosis (Han et al., 2006; Zhang et al., 2019).	+3.4	5.6E-03	+4.5	1.7E-03

Protein name	**Gene**	**Direct or potential relevance to endometriosis**	**Protein FC** (ecESC vs control euESC)	**p-value** (ecESC vs control euESC)	**Protein FC** (ecESC vs endometriotic euESC)	**p-value** (ecESC vs endometri-otic euESC)
Procollagen-lysine, 2-oxoglutarate 5-dioxygenase 2	PLOD2	ECM reorganization under hypoxia (Gilkes et al., 2013).	+3.4	1.1E-03	+5.4	8.2E-05
Protein-lysine 6-oxidase	LOX	Increased invasiveness and migration, upregulated in endometriosis (Ruiz et al., 2015).	+1.5	6.1E-01	+4.1	1.1E-02
Plastin-2	LCP1	Promotion of tumour metastasis (Pillar et al., 2019), down-regulated in endometriosis (Barragan et al., 2016).	–1.8	1.9E-01	–4.9	8.4E-04
Heat shock-related 70 kDa protein 2	HSPA2	Down-regulated in response to hypoxia in epidermal keratinocytes (Scieglinska and Krawczyk, 2015).	–3.4	3.4E-03	–4.3	1.1E-03
Caldesmon	CALD1	NA	–3.4	3.6E-03	–4.2	1.6E-03
Endoplasmic reticulum metallopeptidase 1	ERMP1	Down-regulation decreases HIF1A (Grandi et al., 2016).	–3.9	8.8E-03	–5.1	3.4E-03
Nicotinate-nucleotide pyro-phosphorylase [carboxylating]	QPRT	NAD^+ biosynthesis, anti-apoptotic effects (Ullmark et al., 2017).	–4.2	2.3E-02	–4.5	2.1E-02
EH domain-containing protein 3	EHD3	Behaves as a tumour suppressor in addition to regulation of endocytic transport (Chukkapalli et al., 2014).	–4.5	2.6E-05	–1.8	4.3E-02
Laminin subunit alpha-5	LAMA5	Promotion of angiogenesis (Zhang et al., 2018).	–4.6	1.3E-04	–3.0	3.1E-03
Interferon-induced transmembrane protein 3	IFITM3	Expressed in ovarian endometriomas (Fraunhoffer et al., 2015).	–4.7	1.3E-03	–2.3	8.2E-02
Protein Wnt-5a	WNT5A	Oncogenic and tumour suppressive activity (Zhu et al., 2014b).	–4.7	1.2E-03	–4.0	3.9E-03

Protein name	Gene	Direct or potential relevance to endometriosis	Protein FC (ecESC vs control euESC)	p-value (ecESC vs control euESC)	Protein FC (ecESC vs endometriotic euESC)	p-value (ecESC vs endometri-otic euESC)
Inositol-3-phosphate synthase 1	ISYNA1	Knock-down increases chemoresistance, mediates p53-dependent tumour suppression in HCT116 cells (Koguchi et al., 2016).	–4.7	4.3E-05	–3.3	8.5E-04
Inactive serine protease PAMR1	PAMR1	Tumor suppressor (Lo et al., 2015).	–4.8	1.2E-03	–7.1	7.7E-04
Tetraspanin-14	TSPAN14	NA	–4.8	2.3E-05	–3.8	2.8E-04
Synaptic vesicle membrane protein VAT-1 homolog-like	VAT1L	NA	–5.1	9.6E-03	–2.7	1.6E-01
Podocalyxin	PODXL	Anti-adhesive activity (Boman et al., 2013)	–5.1	7.9E-04	–3.6	8.0E-03
Protein wntless homolog	WLS	NA	–5.2	2.3E-07	–3.7	4.9E-06
Glutaredoxin-1	GLRX	Antioxidant enzyme, reduces fibrosis (Anathy et al., 2018).	–5.5	1.3E-02	–3.8	6.4E-02
Echinoderm microtubule-associated protein-like 1	EML1	NA	–5.9	9.0E-07	–5.5	2.3E-06
Nestin	NES	Expression dependent on bone morphogenetic pathway signalling (Fazeli et al., 2016).	–6.1	7.5E-04	–4.3	5.6E-03
Caspase-1	CASP1	Mediation of inflammation induced apoptosis (Denes et al., 2012), knockdown enhances cell proliferation (Hu et al., 2010); tumour suppressor (Celardo et al., 2013).	–10.1	1.0E-04	–11.9	1.4E-04
Redox-regulatory protein FAM213A	FAM213A	Promotor hypermethylated in invasive melanoma cells (Koroknai et al., 2019).	–19.3	2.6E-05	–15.1	9.9E-05

Mean fold changes with ANOVA post-hoc p-values are presented. Positive/negative values indicate up-/downregulation in ectopic endometrial stromal cells (ecESCs) relative to eutopic endometrial stromal cells (euESCs). Potential endometriosis-relevant functions have been indicated. Abbreviations: FC – fold change, NA – not available.

5.1.2 Extensive changes in energy metabolism in ecESCs (Study I)

As differences were more pronounced in ectopic vs eutopic comparisons than between eutopic cells from patients and healthy women, we focused further on proteins significantly different in ecESCs. To more effectively mine the data in significantly down- and upregulated protein lists for systems-level themes, we used enriched KEGG Pathway and Gene Ontology Biological Process terms associated with the respective proteins.

Most strikingly among the significantly enriched themes in ecESCs we found evidence for upregulated glycolysis (enrichment FDR < 0.001) and downregulated tricarboxylic acid (TCA) cycle (FDR<0.001) and oxidative phosphorylation (FDR<1×10^{-21}). Proteins associated with these pathways were up- or down-regulated in a coordinated fashion relative to euESCs (Figure 12) which is reminiscent of the Warburg effect described in various tumors. Additionally, key enzymes participating in glycolysis [phosphofructokinases PFKP, PFKL – rate-limiting irreversible step; L-lactate dehydrogenase chain A (LDHA) – regeneration of NAD^+ for anaerobic respiration; pyruvate kinase, tumor isoform (PKM2) – essential for maintaining the Warburg effect in tumors (Christofk et al., 2008)] are all significantly upregulated in ecESCs (FC_{PFKP}=+2.8, p=0.008; FC_{PFKL}=+1.7, p=0.002; FC_{LDHA}=+2.7, p=0.00004; and FC_{PKM2}=+2.0, p=0.007). Furthermore, it was evident that less active glycolytic enzyme isoforms (FC_{PFKM}=–1.6, p=0.001 and FC_{LDHB}=–1.5, p=0.0004) were downregulated in contrast to their active isoforms (Figure 13).

Several terms emerged from KEGG analysis ('focal adhesion', 'tight, gap and adherens junction', 'ECM-receptor interaction', 'leukocyte transendothelial migration') that indicated upregulation of numerous proteins involved in cellular adhesiveness and motility in ecESCs (Figure 14A). Strongest upregulation was seen for different collagens (COL1A1, COL1A2, COL3A1, COL5A1), fibronectin (FN1), spectrin alpha chain, nonerythrocytic 1 (SPTAN1), and cGMP-dependent protein kinase (PRKG1). Differently from the energy metabolism alterations, many of the adhesion and motility involved proteins also seem to show increasing upregulation across control euESC, endometriotic euESC and ecESC.

We also found alterations in immune related proteins in primary ecESCs compared to euESCs. Several antigen peptide transporters (TAP1, TAP2, TAPBP) and major histocompatibility complex subunits (B2M, HLA-A2, -C) are lower, while HLA-A3 subunit is strongly upregulated both in endometriotic euESCs and ecESCs compared to healthy euESCs (FC=+4.5, p=0.02; FC=+5.8, p=0.004), respectively (Figure 14B). Most proteasomal subunits show negligible down-regulation, while subunits involved in the formation of the immunoproteasome (PSME1, –2, –3, PSMB8, –9, –10) are downregulated in a pronounced manner (Figure 14C).

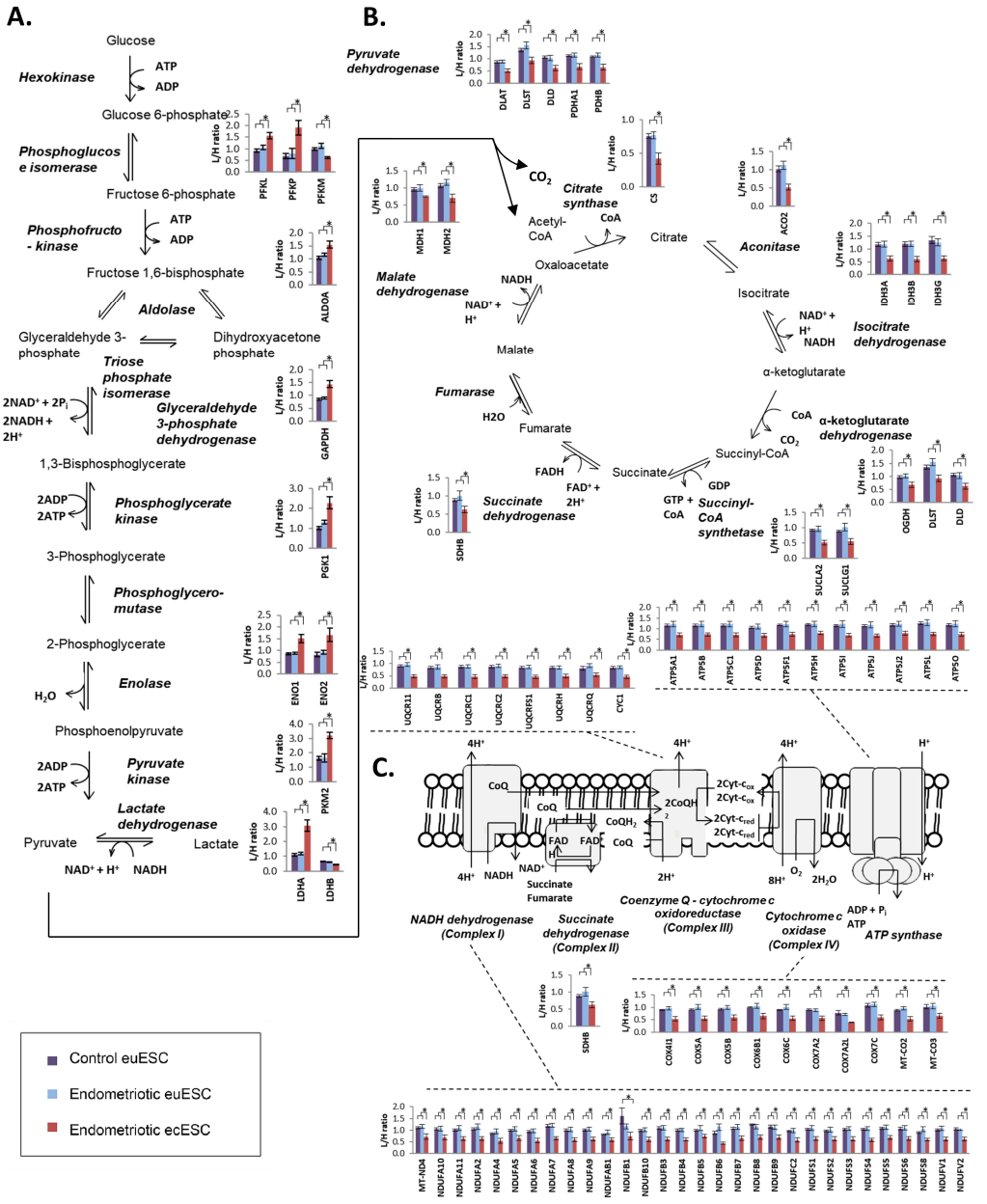


Figure 12. Protein expression level evidence for the Warburg effect in ecESCs characterized by upregulated glycolysis **(A)** and downregulated oxidative metabolism (TCA cycle **(B)** and oxidative phosphorylation **(C)**) under normoxic conditions. Expression levels of different proteins are indicated by mean L/H ratio ± standard error where the light and heavy signals are the sample and standard channels, respectively. * $p<0.05$.

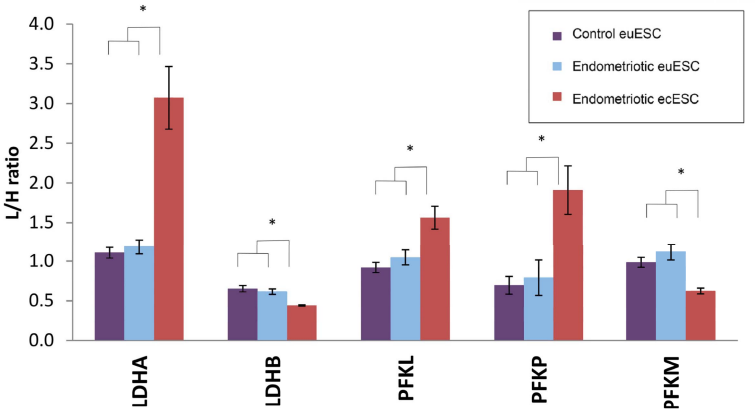


Figure 13. ecESCs upregulate more active glycolytic enzyme isoforms (PFKL, LDHA) over less active ones (LDHB, PFKM). Upregulation is also seen for PFKP. * p<0.05.

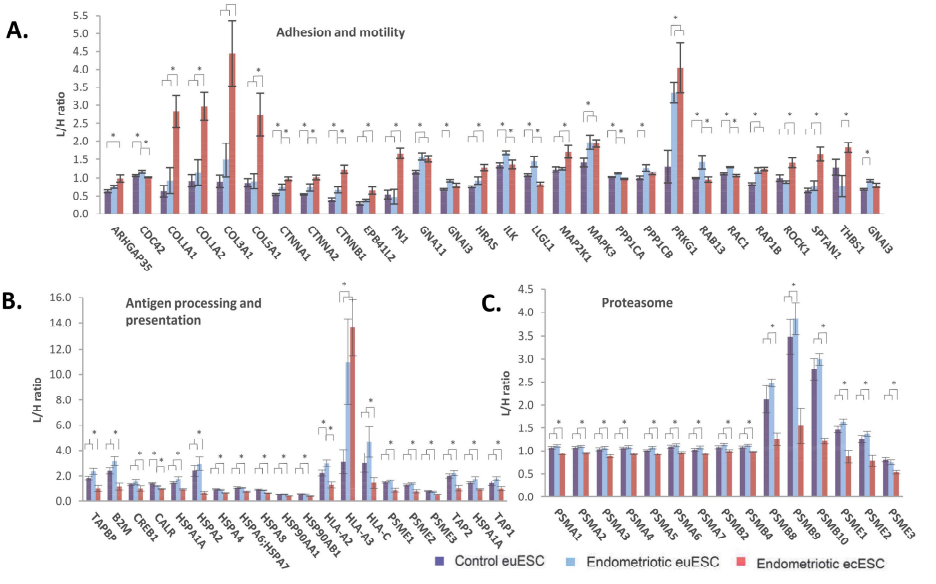


Figure 14. Expression profiles of ANOVA significant proteins of different enriched KEGG themes: **A.** Proteins involved in adhesiveness and motility; **B.** Antigen processing and presentation; **C.** Proteasome subunits. * p<0.05.

5.1.3 Alterations of proteins involved in proliferation, survival and apoptosis in ecESCs (Study I)

In addition to term enrichment analysis we looked at various proteins known to regulate cell survival or apoptosis that are significantly altered (ANOVA q<0.05) across sample groups. Based on *post-hoc* analyses (p<0.05), we found eight downregulated and one upregulated pro-apoptotic (*i.e.* tumor suppressors) proteins, and five upregulated and one downregulated anti-apoptotic (*i.e.* proto-onco- or oncogenes) proteins in ecESCs, whereas only two anti-apoptotic proteins were significantly upregulated in endometriotic euESCs with others showing no significant changes compared to control euESCs (Table 6).

Protein	**Gene**	**FC (p-value),** endometriotic euESC vs control euESC	**FC (p-value),** ecESC vs endometriotic euESC	**FC (p-value),** ecESC vs control euESC	
Apoptosis-associated speck-like protein containing a CARD	ASC	–1.2 (p=0.2)	**–1.5 (p=0.02)**	**–1.8 (p=0.0005)**	**Pro-apoptotic**
Apoptosis-inducing factor 2	AIFM2	+1.5 (p=0.1)	**–1.7 (p=0.009)**	–1.2 (p=0.5)	
Apoptosis regulator BAX	BAX	+1.1 (p=0.5)	**–1.7 (p=0.0000003)**	**–1.6 (p=0.0000007)**	
Caspase-1	CASP1	+1.2 (p=0.9)	**–11.9 (p=0.0001)**	**–10.1 (p=0.0001)**	
Caspase-7	CASP7	–1.1 (p=0.9)	**–2.0 (p=0.004)**	**–2.1 (p=0.001)**	
Cyclin-dependent kinase inhibitor 2A	CDKN2A	+1.3 (p=0.5)	**+2.3 (p=0.009)**	**+3.3 (p=0.0009)**	
Death-associated protein kinase 3	DAPK3	–1.1 (p=0.9)	**–1.8 (p=0.02)**	**–2 (p=0.008)**	
Inactive serine protease PAMR1	PAMR1	+1.5 (p=0.4)	**–7.1 (p=0.0008)**	**–4.8 (p=0.001)**	
Mothers against decapentaplegic homolog 4	SMAD4	+1.1 (p=0.3)	**–1.4 (p=0.002)**	**–1.3 (p=0.04)**	**Anti-apoptotic**
Apoptosis inhibitor 5	API5	+1.0 (p=1.0)	**–1.6 (p=0.0001)**	**–1.6 (p=0.00007)**	
Catenin beta-1	CTNNB1	**+1.7 (p=0.03)**	**+1.9 (p=0.02)**	**+3.2 (p=0.00009)**	
Catenin alpha-1	CTNNA1	**+1.3 (p=0.04)**	+1.3 (p=0.05)	**+1.8 (p=0.0003)**	
Catenin alpha-2	CTNNA2	+1.3 (p=0.11)	**+1.4 (p=0.04)**	**+1.9 (p=0.0007)**	
GTPase HRas	HRAS	+1.2 (p=0.3)	**+1.4 (p=0.04)**	**+1.7 (p=0.001)**	
Tumor protein D53	TPD52L1	+1.9 (p=0.4)	**+4.1 (p=0.03)**	**+7.9 (p=0.005)**	

Abbreviations: FC – fold change, euESC – eutopic endometrial stromal cell, ecESC – ectopic endometrial stromal cell.

5.1.4 The main inducer of hypoxia response is upregulated in ecESCs (Study I)

As our proteomics data did not cover the hypoxia-inducible factor 1-alpha (HIF1A), we used qRT-PCR to detect its expression in eu- and ecESCs. We also included various other glycolytic and oxidative phosphorylation enzymes genes (*COX6C*, *UQCRC1*, *-2*, *NDUFB6, ATP5H, PGK1, ENO1, PFKP, PFKL*) to validate the accuracy of our proteomics measurements. We did not use *GAPDH* as a reference gene, as the protein level data indicated that it is upregulated in ecESCs ($FC_{ecESC/end.euESC}$=+1.6, p=0.002; $FC_{ecESC/euESC}$=+1.7 p=0.0006), and *HPRT1* was used instead ($FC_{ecESC/end.euESC}$=+1.1, p=0.6 and $FC_{ecESC/euESC}$=–1.1 p=0.9). Most of the primary cells used in qRT-PCR experiment were from an independent group of patients (4/5 patients) than the one used in proteomics.

qRT-PCR showed that *HIF1A* is indeed significantly upregulated in ecESCs ($FC_{ecESC/end.euESC}$=+2.3, p=0.0002) (Table 7), which is consistent with a Warburg effect in ecESCs. All other included metabolic gene mRNAs have similar significant changes to protein levels with the exception of NDUFB6 and ATP5H, which did not reach significance on the mRNA level.

Table 7. mRNA level changes of selected glycolytic and oxidative phosphorylation enzymes, and HIF1A, in ecESC *vs* endometriotic euESC. Positive/negative values indicate up-/downregulation in ecESCs relative to euESCs. Significantly ($p<0.05$) differently expressed genes are in bold.

Glycolysis			***Oxidative phosphorylation***		
Gene	**mRNA FC (ectopic/eutopic)**	**p-value**	**Gene**	**mRNA FC (ectopic/eutopic)**	**p-value**
PGK1	**2.08**	**0.001**	***COX6C***	**–1.72**	**0.02**
ENO1	**2.07**	**0.002**	***UQCRC1***	**–1.91**	**0.004**
PFKP	**4.89**	**0.0003**	***UQCRC2***	**–1.42**	**0.003**
PFKL	**1.35**	**0.01**	*NDUFB6*	–1.1	0.3
HIF1A	**2.32**	**0.0002**	*ATP5H*	–1.19	0.2

FC – fold change, euESC – eutopic endometrial stromal cell, ecESC – ectopic endometrial stromal cell.

5.1.5 ecESCs have attenuated mitochondrial respiration (Study I)

Next, we used live-cell high-resolution respirometry to determine whether the expressional level changes translate directly into reduced oxygen consumption in ecESCs compared to euESCs. We found that in routine state ecESCs compared to the same patient's euESCs respire at a reduced level (ΔV_r=–2.5±0.9 nmol/(min × mg), p=0.01) (Figure 15A) and have less oxygen consumption due to oxidative phosphorylation ($\Delta(V_r-V_{Oly})/(V_{r,ecESC}-V_{Oly,ecESC})$ = –33.7±15.2%, p=0.046)

(Figure 15B). Notably strong reductions are observed for maximal respiratory potential of ecESCs ($\Delta V_{FCCPmax}$= –8.3±1.8 nmol/(min x mg), p=0.0004) and maximal respiratory chain capacity compared to euESCs ($\Delta(V_{FCCP}-V_{AntA})/(V_{FCCP,ecESC}- V_{AntA,ecESC})$ = –44.7±5.5%, p=0.0006) (Figure 15B). The first parameter reflects the difference of oxygen consumption when the mitochondrial proton gradient is disrupted/uncoupled and the electron chain complexes are maximally stimulated, while the second parameter expresses maximal respiration difference of ecESCs compared to euESCs where oxygen consumption is attributable to oxidative phosphorylation. There was also less respiration due to proton leakage in ecESCs ($\Delta(V_{Oly}-V_{AntA})/(V_{Oly,ecESC}-V_{AntA,ecESC})$= =–40.6±9.2%, p=0.006).

Finally, we also compared activities of several enzymes important in energy metabolism of ecESCs with the same patient euESCs. We found evidence for lowered activity for citrate synthase (–32.2±9.2%, p=0.02), adenylate kinase (–43.6±1.5%, p=0.0001) and creatine kinase (–50.3±10.8%, p=0.02).

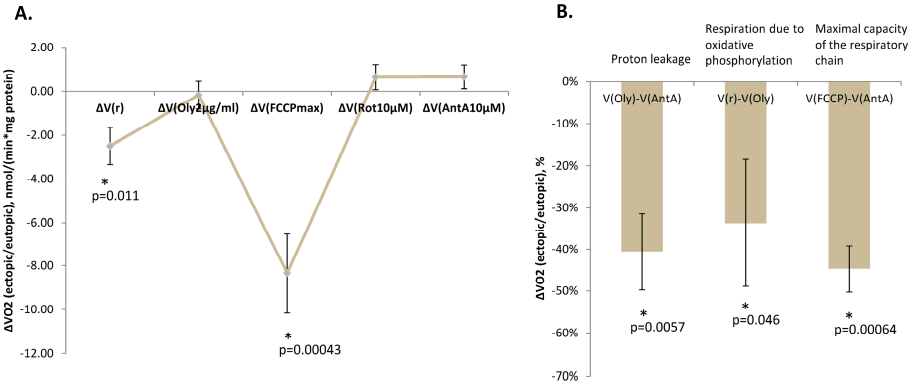


Figure 15. EcESCs respire at an attenuated rate and have lower respiratory capacity compared to euESCs. **(A)** Difference of ecESC versus euESC respiration is shown in the presence of and in the absence (V_r) of various respiration affecting poisons (Oly, FCCP, Rot, AntA). **(B)** Relative changes of ecESC versus euESC respiratory parameters (proton leakage, respiration due to oxidative phosphorylation and maximal respiratory chain capacity) are presented. Data are presented as mean change (ectopic *versus* eutopic) ± standard error of the mean (SEM). Significance with respective p-values are indicated (*). Abbreviations: V_r – routine oxygen consumption, Oly – oligomycin, ATP synthase inhibitor, FCCP max – maximal respiration induced by the ionophore FCCP, Rot – rotenone, complex I inhibitor, AntA – antimycin A, complex III inhibitor.

5.1.6 Lack of endometriosis-specific protein markers in pooled and immunodepleted blood plasma (Study I, unpublished results)

We used comprehensive proteomics analysis also on blood plasma samples of women with and without endometriosis to reveal endometriosis-specific protein markers that could be used as non-invasive markers for endometriosis diagnostics.

To maximize the number of individuals for studying potential endometriosis-specific plasma biomarkers without excessively inflating total LC/MS/MS measuring time, we used pooled plasma samples of endometriosis patients (24 pools, 119 women) and controls (12 pools, 53 women) (Figure 7). Our endometriosis cohort contained follicular and luteal phase plasma samples from women with stage I–II and III–IV endometriosis. To rule out unspecific markers (*i.e.* may be altered in other gynecological conditions) that can emerge when using healthy women without any clinical complaints as controls, the control group consisted of women undergoing laparoscopy because of endometriosis-like symptoms, but to whom endometriosis diagnosis was not confirmed. This also helped to rule out any controls who could have asymptomatic endometriosis.

As plasma is dominated by albumin and other highly abundant proteins, which render the depth of detection very shallow, we decided to use immunodepletion of the 14 most abundant proteins by MARS-14 (Human 14 Multiple Affinity Removal System®) in blood plasma (Figure 16A). This approach enabled us to significantly increase the number of different proteins detectable by shotgun proteomics from 260 (with 3,026 different peptide sequences) with non-depleted samples to 964 (13,775 peptides) with depleted samples. Nevertheless, despite successfully depleting for the highest 14 proteins (Figure 16A 'MARS-14 retained

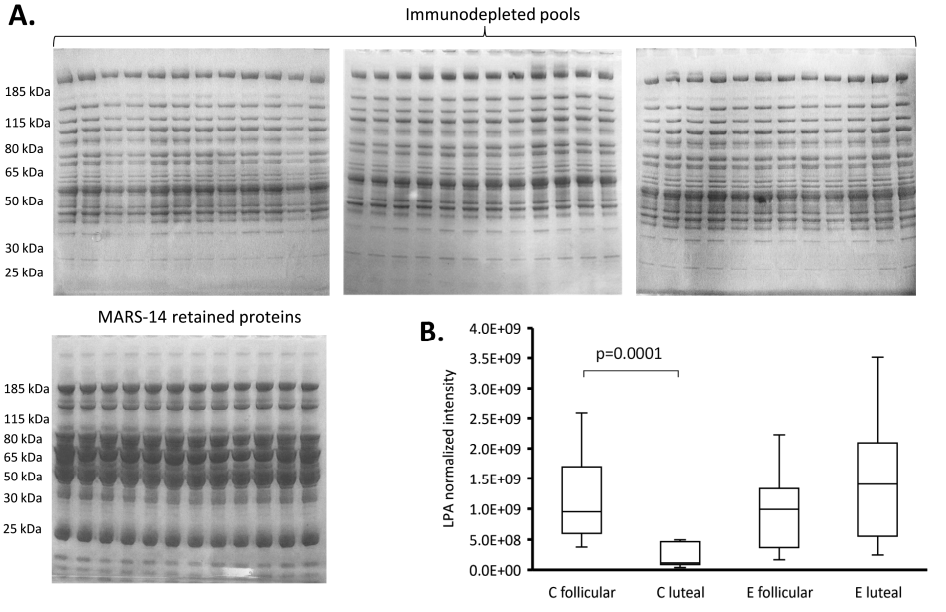


Figure 16. A. SDS-PAGE analysis of immunodepleted plasma pools (*top*) and MARS-14 column retained proteins (*bottom left*). **B.** LPA shows a trend for down-regulation in luteal phase control samples compared to endometriosis group, where phase-specific change is not observable. Although, a highly significant change is observable when looking at LPA selectively, the effect is merely trending ($q<0.1$) for significance when corrected for multiple testing in the statistical analysis of the proteomics list.

proteins') the resulting samples still contained another level of very abundant and non-changing proteins (such as A1BG, APOB, APOH, C4B, CFB, CFH, CP, GC, HPX, ITIH1, ITIH2, ITIH4, SERPINA3, SERPINC1; based on LC/MS/MS) among the top represented proteins (Figure 16A 'Immunodepleted pools').

ANOVA analysis based on endometriosis and control grouping revealed 4 proteins that had differences between groups (complement receptor type 2, p=0.001; sulfhydryl oxidase, p=0.002; platelet-activating factor acetylhydrolase, p=0.003; and proteoglycan 4, p=0.005), but none of these remained significant or trending for significance after multiple testing correction (q>0.1). Subgrouping into additional groups based on endometriosis stages and/or menstrual cycle phases did not result in any other significant proteins, although, apolipoprotein(a) (LPA) showed a trend for significance (q=0.07) when analyzing endometriosis and control groups based on cycle phases (follicular versus luteal). In control samples, LPA was lower in luteal compared to follicular phase (FC=–7.0), whereas there was no difference in the endometriosis group (Figure 16B). As the discovery experiment using immunodepletion and sample pooling revealed neither sufficiently robust nor endometriosis-specific markers, we did not pursue further investigations into plasma-based protein biomarkers.

5.2 Assessment of endometrial receptivity through uterine fluid proteomics (Studies II–IV)

5.2.1 Profiling of uterine fluid protein composition from early to mid-secretory phase (Study II)

To find potential new endometrial receptivity markers, we first sought to measure the entire uterine fluid proteome of six healthy fertile women and detect the changes from ESE to MSE phase with discovery proteomics, and, then sieve and evaluate the most useful markers with targeted proteomics.

Due to the presence of very high levels of serum albumin in uterine fluid (Figure 17A), samples were gel-fractionated to minimize the suppressive effect that albumin peptides can have on lower abundant co-eluting peptides. Measuring the samples in this manner enabled us to identify and quantify 36,171 peptides belonging to 3,158 different proteins (FDR<1%; 2,758 proteins with ≥2 unique peptides) or protein groups of which we filtered out 2,196 that met the criteria for statistical analysis (≥50% valid values per group).

Based on UniProt 'cellular localization' term enrichment, uterine fluid contains more exosomal, extracellular, extracellular matrix-associated and plasma membrane proteins compared to glandular epithelium, but also more cytosolic and endoplasmic reticulum luminal proteins (Figure 17B). It is noteworthy that many of these proteins have multiple localizations, particularly proteins that are also partitioned into extracellular vesicles (~47.0% of cytosolically annotated

proteins have also exosomal annotation). The fluid is less represented by mitochondrial, nuclear and endoplasmic reticulum membrane proteins compared to glandular cells.

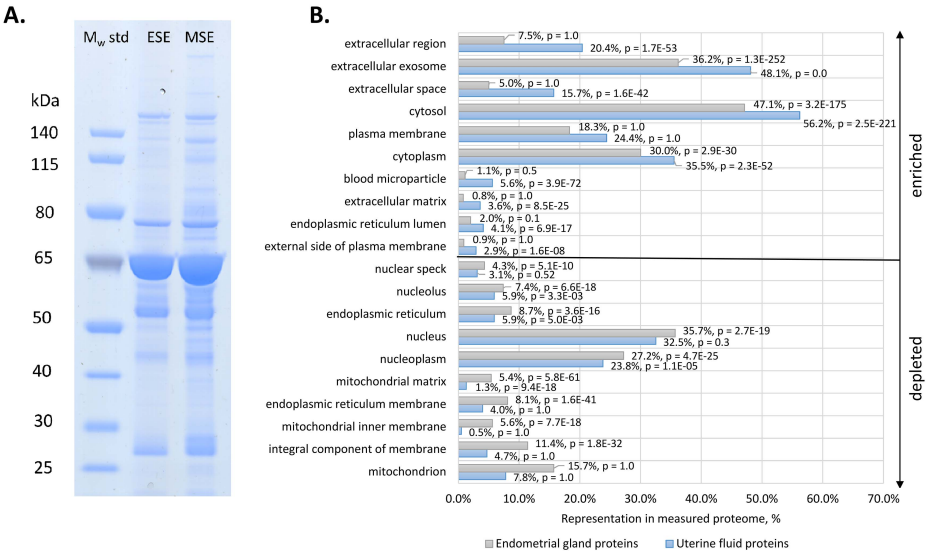


Figure 17. A. Uterine fluid proteome is dominated by high levels of albumin (band at ~66.5 kDa). ESE and MSE samples of a single healthy fertile volunteer are presented. **B.** Characterization of uterine fluid proteome composition. Top ten UniProt 'cellular localization' terms showing the greatest magnitude of increase or decrease in the uterine fluid compared to endometrial glandular tissue. P-values of enrichment relative to the entire background human proteome have been indicated. Abbreviations: M_w std – molecular weight standard.

When looking at the quantitative differences between ESE and MSE we found that during the transition 367 proteins changed significantly ($q \leq 0.05$, FC range: –15.2 to +60.9), from which 185 proteins had at least twofold change (Figure 18). The genes for 20 of these significantly changing proteins are also included in the ERA® test (*ANXA4*, *ARHGDIB*, *ATP6V1A*, *COMP*, *CORO1A*, *CRISP3*, *CTNNA2*, *DPP4*, *ECM1*, *FGB*, *GBP2*, *HABP2*, *HMHA1*, *MMP26*, *NNMT*, *PAEP*, *POSTN*, *PSMB10*, *SFRP4*, *TCN1*) (Diaz-Gimeno et al., 2011a). With the exception of MMP26 and HABP2, majority of proteins show abundance changes in the same direction as measured on the endometrial tissue mRNA level. The overall overlap of genes used in ERA® (n=238) and uterine fluid proteins subjected to statistical analysis in our data (n=2196) is 54.

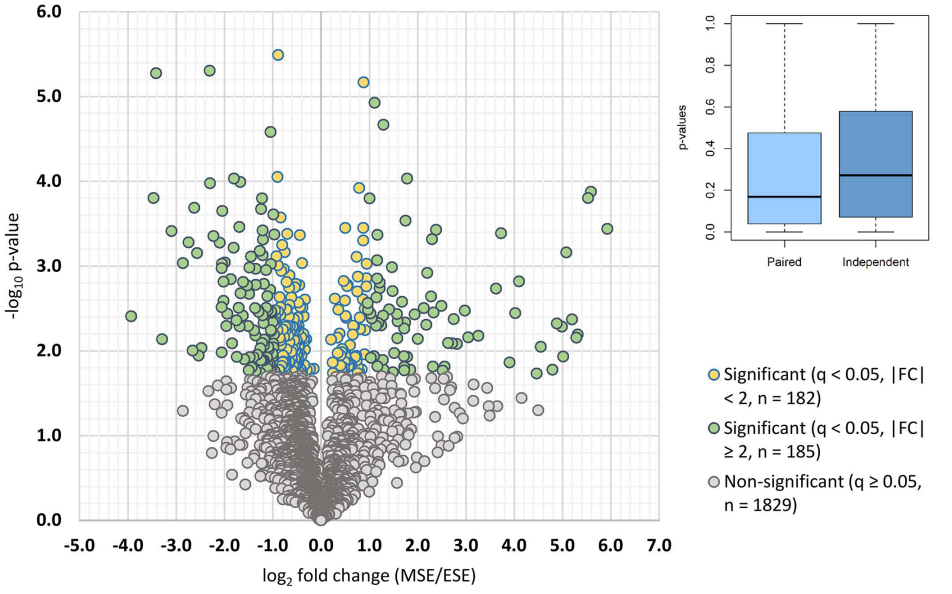


Figure 18. Volcano plot (*left*) summarizing the differences between ESE and MSE proteomes. Data are presented as $\log_2$ of fold changes from ESE to MSE against $\log_{10}$ of p-values. Proteins whose abundance change remained statistically significant after correcting for multiple testing have been color-coded yellow (< 2-fold difference) and green (≥ 2-fold difference). The p-value distributions (*right*) of either using a paired or independent test show that the within-subjects variability is less than between subjects.

As our study used paired samples from the same individual, we also noted that for many proteins the abundances in each time point had more variability (*i.e.* between-subjects variability) than the individual ESE to MSE changes (within-subjects variability). This underlies that paired in contrast to independent design is more effective for finding receptivity-specific changes. This is also evident from the p-value distributions where the paired one is shifted toward lower values compared to p-values obtainable with an independent t-test (Figure 18).

Gene Ontology 'Biological process' term enrichment analysis of MSE significantly upregulated proteins indicated that relative to the entire human proteome there is enriched number of proteins participating in immune response, coagulation and sugar metabolism (Figure 19A). MSE downregulated analysis showed higher representation of proteins associated with DNA replication, mRNA splicing and endoplasmic reticulum to Golgi vesicle mediated transport (Figure 19B).

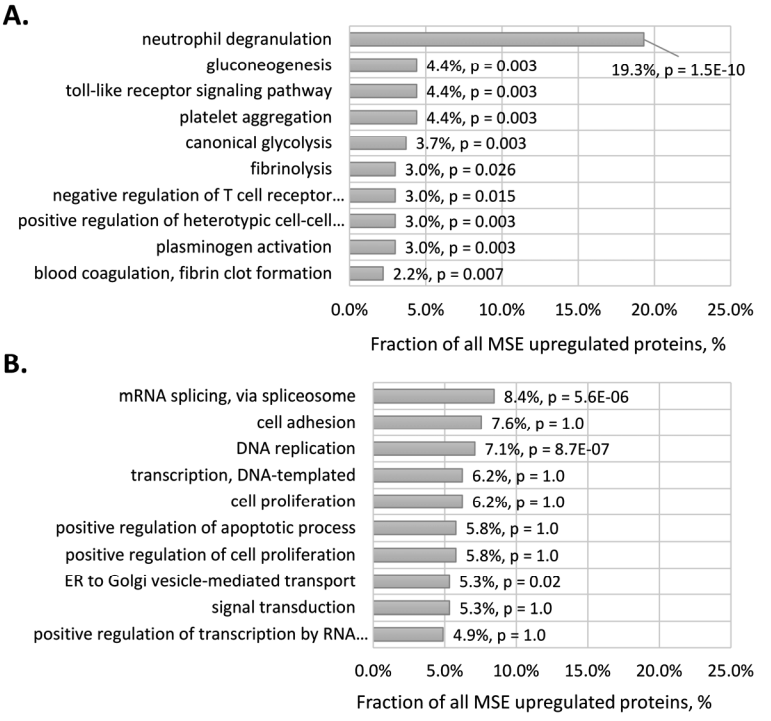


Figure 19. A. Top ten enriched Gene Ontology 'Biological process' terms relative to entire human proteome background among MSE upregulated proteins. **B.** Top ten enriched Gene Ontology 'Biological process' terms relative to the entire human proteome background among MSE downregulated proteins. Enrichment p-values have been indicated.

5.2.2 Proteins previously not associated with endometrial receptivity (Study II)

To determine if proteins with a very large effect size ($|FC| \geq 5$, $q<0.05$, n=45) in our uterine fluid discovery data (Table 8) have been previously described in the context of human endometrial receptivity, we performed a comprehensive literature search. We found that 32 out of the 45 proteins/genes from this set have been reported in the context of endometrial functioning and/or receptivity. These studies were mostly conducted on the transcript level using tissue biopsies. Therefore, 13 proteins (GRN, MPO, ELANE, SLC34A2, LCN2, PARP4, CAND2, ISYNA1, PAMR1, PALLD, CDH11, ITGA6 and COL7A1) to the best of our knowledge are novel and have not previously been implicated in uterine receptivity in human studies.

Table 8. Proteins with more than fivefold abundance difference between ESE and MSE uterine fluid in discovery LC/MS/MS. Proteins shown in bold were also positively validated ($p<0.05$) by targeted MS as described in section 5.2.4.

Protein name	Gene name	UniProt ID	Fold change $FC_{MSE/ESE}$* (q-value)	Reference	Analyzed endometrial material in prior studies	Evidence level for gene expression in prior studies
Nicotinamide N-methyltransferase	NNMT	P40261	+60.9 (0.011)	(Allegra et al., 2012; Chan et al., 2013; Garrido-Gomez et al., 2011)	Uterine fluid; tissue; *in vitro* cell line	mRNA, protein
Glycodelin	PAEP	P09466	+48.3 (0.008)	(Casado-Vela et al., 2009; Garrido-Gomez et al., 2011; Kao et al., 2002)	Tissue; *in vitro* cell line; uterine fluid	mRNA, protein
Transcobalamin-1	TCN1	P20061	+46.4 (0.008)	(Chan et al., 2013; Diaz-Gimeno et al., 2011b)	Uterine fluid; tissue	mRNA
Sulfate transporter	SLC26A2	P50443	+39.9 (0.03)	(Qiao et al., 2008)	Tissue	mRNA
Ectonucleotide pyrophosphatase/ phosphodiesterase 3	ENPP3	O14638	+39.1 (0.031)	(Chen et al., 2018)	Tissue	protein
Granulins	GRN	P28799	+36.8 (0.025)	NA	NA	NA
Stanniocalcin-1	STC1	P52823	+33.9 (0.014)	(Allegra et al., 2009)	Tissue	mRNA
Dipeptidyl peptidase 4	DPP4	P27487	+32.4 (0.04)	(Riesewijk et al., 2003)	Tissue	mRNA
Myeloperoxidase	MPO	P05164	+31.8 (0.027)	NA	NA	NA
Complement decay-accelerating factor	CD55	P08174	+29.6 (0.027)	(Franchi et al., 2008; Mirkin et al., 2005)	Tissue	mRNA, protein
Neutrophil elastase	ELANE	P08246	+27.8 (0.046)	NA	NA	NA
Aldehyde dehydrogenase family 1 member A3	ALDH1A3	P47895	+23.5 (0.036)	(Allegra et al., 2009)	Tissue	mRNA
Sodium-dependent phosphate transport protein 2B	SLC34A2	O95436	+22.1 (0.048)	NA	NA	NA

Protein name	Gene name	UniProt ID	Fold change $FC_{MSE/ESE}$* (q-value)	Reference	Analyzed endometrial material in prior studies	Evidence level for gene expression in prior studies
Neutrophil gelatinase-associated lipocalin	LCN2	P80188	+17.2 (0.018)	NA	NA	NA
Mesothelin	MSLN	Q13421	+16.3 (0.025)	(Dassen et al., 2007)	*In vitro* cell line	mRNA
Platelet glycoprotein 4	CD36	P16671	+15 (0.042)	(Bhagwat et al., 2013)	Tissue; *in vitro* cell line	mRNA, protein
Poly [ADP-ribose] polymerase 4	PARP4	Q9UKK3	+13.3 (0.011)	NA	NA	NA
Cartilage oligomeric matrix protein	COMP	P49747	+12.3 (0.019)	(Bhagwat et al., 2013; Chan et al., 2013)	Uterine fluid; tissue	mRNA, protein
Cathepsin B	CTSB	P07858	+9.6 (0.03)	(Garrido-Gomez et al., 2011)	*In vitro* cell line	protein
Insulin-like growth factor-binding protein 7	IGFBP7	Q16270	+8.3 (0.031)	(Dominguez et al., 2003)	Tissue	mRNA, protein
Homogentisate 1,2-dioxygenase	HGD	Q93099	+7.9 (0.024)	(Labarta et al., 2011)	Tissue	mRNA
Ribonuclease T2	RNASET2	O00584	+7.1 (0.034)	(Labarta et al., 2011)	Tissue	mRNA
Interferon-induced guanylate-binding protein 2	GBP2	P32456	+6.7 (0.025)	(Chan et al., 2013)	Tissue, uterine fluid	mRNA
Branched-chain amino acid amino-transferase, cytosolic	BCAT1	P54687	+6.6 (0.034)	(Haouzi et al., 2009; Scotchie et al., 2009b)	Tissue; uterine fluid	mRNA, protein
Cysteine-rich secretory protein 3	CRISP3	P54108	+6.2 (0.034)	(Diaz-Gimeno et al., 2011b; Evans et al., 2015)	Tissue; uterine fluid	protein
Polymeric immunoglobulin receptor; Secretory component	PIGR	P01833	+5.9 (0.044)	(Haouzi et al., 2009; Scotchie et al., 2009b)	Tissue; uterine fluid	mRNA, protein

Protein name	Gene name	UniProt ID	Fold change $FC_{MSE/ESE}$* (q-value)	Reference	Analyzed endometrial material in prior studies	Evidence level for gene expression in prior studies
Interleukin-6 receptor subunit beta	IL6ST	P40189	+5.7 (0.046)	(Tapia-Pizarro et al., 2014)	Tissue	mRNA
Mitogen-activated protein kinase kinase kinase 5	MAP3K5	Q99683	+5.7 (0.024)	(Tseng et al., 2010)	Tissue	mRNA
Major vault protein	MVP	Q14764	+5.2 (0.011)	(Rai et al., 2010b)	Tissue	protein
Matrix metalloproteinase-26	MMP26	Q9NRE1	+5.0 (0.025)	(Qiao et al., 2008)	Tissue	mRNA
Amiloride-sensitive amine oxidase [copper-containing]	AOC1	P19801	*only MSE***	(Kao et al., 2002)	Tissue	mRNA
Syntenin-2	SDCBP2	Q9H190	*only MSE***	(Chan et al., 2013; Greening et al., 2016)	*In vitro* cell line; uterine fluid	protein, mRNA
Cullin-associated NEDD8-dissociated protein 2	CAND2	O75155	*only ESE***	NA	NA	NA
Inositol-3-phosphate synthase 1	ISYNA1	Q9NPH2	–5.6 (0.036)	NA	NA	NA
Inactive serine protease PAMR1	PAMR1	Q6UXH9	–5.8 (0.04)	NA	NA	NA
Palladin	PALLD	Q8WX93	–5.9 (0.014)	NA	NA	NA
Periostin	POSTN	Q15063	–6.2 (0.01)	(Chan et al., 2013; Kao et al., 2002)	Uterine fluid; tissue	mRNA
Syndecan-2	SDC2	P34741	–6.3 (0.037)	(Haouzi et al., 2011)	Tissue	mRNA
Membrane-associated progesterone receptor component 1	PGRMC1	O00264	–6.7 (0.012)	(Garrido-Gomez et al., 2014)	Tissue	mRNA, protein
Cadherin-11	CDH11	P55287	–7.2 (0.015)	NA	NA	NA

Protein name	Gene name	UniProt ID	Fold change $FC_{MSE/ESE}$* (q-value)	Reference	Analyzed endometrial material in prior studies	Evidence level for gene expression in prior studies
Progesterone receptor	PGR	P06401	–8.5 (0.011)	(Chan et al., 2013; Lessey et al., 1996)	Uterine fluid; tissue	mRNA, protein
Secreted frizzled-related protein 1	SFRP1	Q8N474	–9.8 (0.032)	(Chan et al., 2013)	Uterine fluid	mRNA
Integrin alpha-6	ITGA6	P23229	–10.7 (0.002)	NA	NA	NA
Collagen alpha-1(VII) chain	COL7A1	Q02388	–11.1 (0.008)	NA	NA	NA
Secreted frizzled-related protein 4	SFRP4	Q6FHJ7	–15.2 (0.025)	(Abu-Jawdeh et al., 1999; Fitzgerald et al., 2018a)	Tissue; uterine fluid	mRNA, protein

* Positive value indicates upregulation in MSE relative to ESE, negative value indicates down-regulation in MSE relative to ESE.
** Protein only detected in either MSE or ESE.
NA – not available, FC – fold change.

5.2.3 Correlation between uterine fluid and endometrial tissue gene expression (Studies III and IV)

We were also interested to determine whether the dynamics observed in uterine fluid proteins is in correlation with gene expression in the endometrial tissue. In conjunction with our collaborators, we chose STC1 and ENPP3 from the 45 most differentially abundant fluid proteins to examine their correlation with tissue expression (Table 8). Both proteins are strongly upregulated in uterine fluid of fertile women during opening of the WOI (Figure 20); nevertheless, the localizations of STC1 and ENPP3 have not been characterized in the human endometrium.

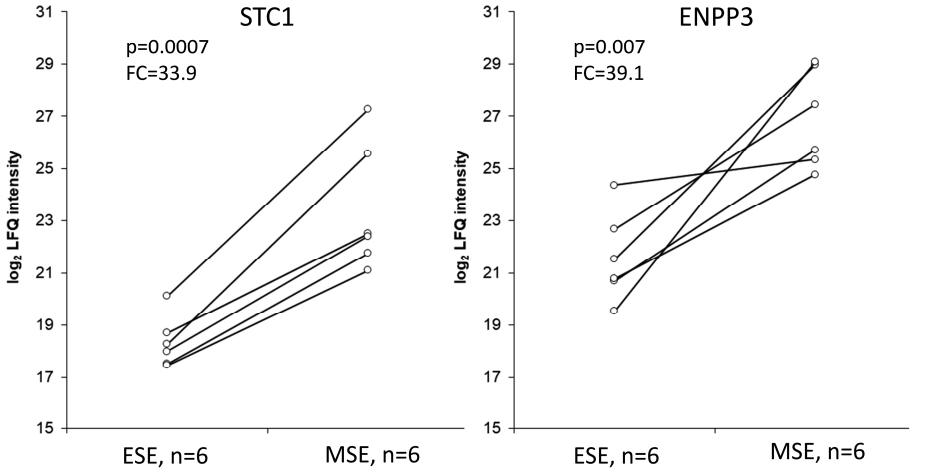


Figure 20. The levels of STC1 and ENPP3 increase in uterine fluid during the mid-secretory phase when the endometrium becomes receptive for embryo implantation. Normalized log_2-transformed label-free (LFQ) protein intensities of paired samples are shown on the y-axis. ESE – early secretory phase, MSE – mid-secretory phase.

ENPP3 mRNA was detectable both in endometrial stromal and glandular compartments (Figure 21A). In the glands, ENPP3 protein is upregulated in the mid- and late secretory phase compared to the proliferative phase (Figure 21B), mirroring the abundance differences also seen in the fluid. Interestingly, immunohistochemistry staining of endometrial tissue showed protein expression only in the apical region of the glandular epithelium despite the transcription seen in stroma (Figure 21C).

STC1 mRNA expression was studied only in whole tissue, where it showed average increase in the ESE and MSE compared to the proliferative phase, however, this was statistically non-significant (Figure 22A). Staining of MSE endometrium showed that STC1 is ubiquitously expressed in the endometrial tissue with positive staining in stromal cells, glandular and luminal epithelial cells (Figure 22B).

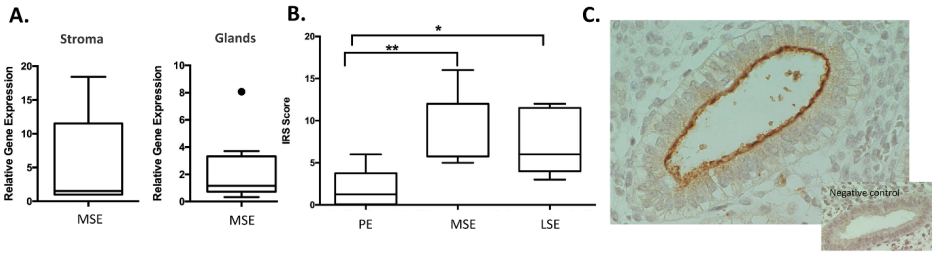


Figure 21. ENPP3 expression in endometrial tissue. **A.** in mid-secretory phase, ENPP3 mRNA is expressed both in endometrial stroma and glands. **B.** ENPP3 protein is upregulated in the mid- and late secretory versus the proliferative phase. **C.** Mid-secretory ENPP3 shows staining only in the apical region of the glandular epithelium. * $p<0.05$, ** $p<0.001$, IRS – immunoreactive score, PE – proliferative phase, MSE – mid-secretory phase, LSE – late secretory phase.

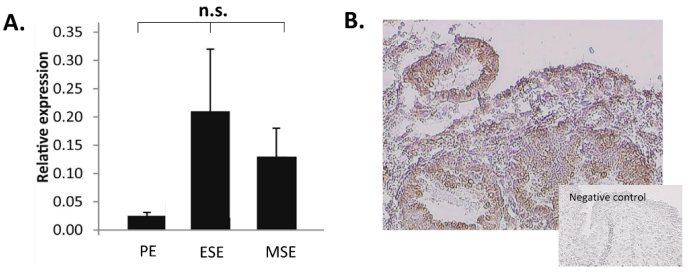


Figure 22. A. Whole endometrial tissue STC1 mRNA is expressed throughout the menstrual cycle with varying levels. **B.** Mid-secretory STC1 protein is expressed in endometrial stromal and glandular cells. PE – proliferative phase, ESE – early secretory phase, MSE – mid-secretory phase, n.s. – non-significant.

5.2.4 Validation of uterine fluid discovery data with targeted proteomics (Study II)

To determine which of the 45 proteins listed in Table 8 would be robust enough for potential further diagnostic development, we assembled a targeted MS-assay using suitable peptides of the proteins identified in the discovery experiment. For the validation, new healthy controls (n=11) were recruited and we also included a cohort of RIF-patients for whom endometrial-factor RIF was suspected (n=29). During the clinical collection of uterine lavages, it was observed that the yield and protein concentration tended to vary. Therefore, to counteract this effect, peptides from EEF1A1 protein were included into the targeted assay as intensity normalization peptides, as EEF1A1 showed nearly constant level across samples in the discovery experiment ($FC_{MSE/ESE}$=1.0, p=0.99, CV=0.7%). Albeit, EEF1A1 is present in erythrocytes (Bryk and Wisniewski, 2017) and based on visual observations, uterine fluid samples tend to have varying levels of contamination

by hemolyzed blood. We did observe fluctuating levels of hemoglobin alpha 1 chain (HBA1) peptides in the validation samples (Figure 23A), but HBA1 was found to explain only ~9% of the variability of EEF1A in the fluid (Figure 23B). Therefore, samples were not excluded based on hemolyzed blood contamination, as it is somewhat unavoidable in the clinical setting (Boomsma et al., 2009a), and by our results does not appear to contribute to the overall EEF1A1 found in the uterine fluid.

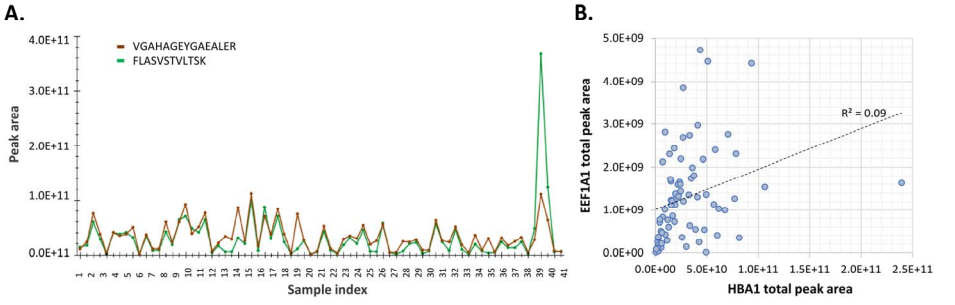


Figure 23. A. Peak areas of HBA1 peptides from sample to sample show varying levels of hemolyzed blood in different uterine lavages. **B.** Correlation between summed peak areas of EEF1A1 and HBA1 peptides.

Out of the 45 proteins selected for the validation phase of the experiment, 38 were confirmed as significantly different ($p<0.05$) between control ESE and MSE samples of new recruits. All of the significant proteins also had abundance differences in the same direction as observed in the discovery part of the study. The targeted MS data distinctly separate ESE and MSE groups in PCA analysis (Figure 24A). Seven proteins (CAND2, CD36, GBP2, IGFBP7, IL6ST, ITGA6, PIGR) did not reach statistical significance in the validation. Overall, we considered 38 out of the 45 significantly changing proteins as validated and sufficiently robust for further evaluation.

5.2.5 Altered window of implantation in women with repeated implantation failure (Study II)

Our validation measurements also included women with RIF (n=29) for whom endometrial-factor implantation defects are suspected. We hypothesized that during the mid-secretory phase RIF women would show alterations in the receptivity markers we chose for validation. Plotting the three different groups (control ESE and MSE with RIF MSE) by their primary components indicated three discernable groups with a partial overlap between control MSE and RIF MSE (Figure 24B). However, over half of the validated proteins (21/38) displayed similar levels between control ESE and RIF MSE instead. This was also reflected in PCA when plotting the high end of the most significantly

different ($p<0.005$) proteins between control MSE and RIF MSE where most overlap is shifted to control ESE and RIF MSE (Figure 24C).

The uterine fluid protein signature consisting of the 21 proteins with ESE-like expression (Figure 24D) thus seems to indicate potential displacement of the window of implantation in our RIF cohort. Nevertheless, not all of the validated proteins from the control ESE and RIF MSE are at the same level, as 17 out of the 38 proteins (ALDH1A3, BCAT1, CDH11, COL7A1, COMP, HGD, ISYNA1, MAP3K5, PAEP, PALLD, PAMR1, PARP4, PGMRC1, POSTN, SDC2, SFRP1, SFRP4) still display similar levels between control MSE and RIF MSE.

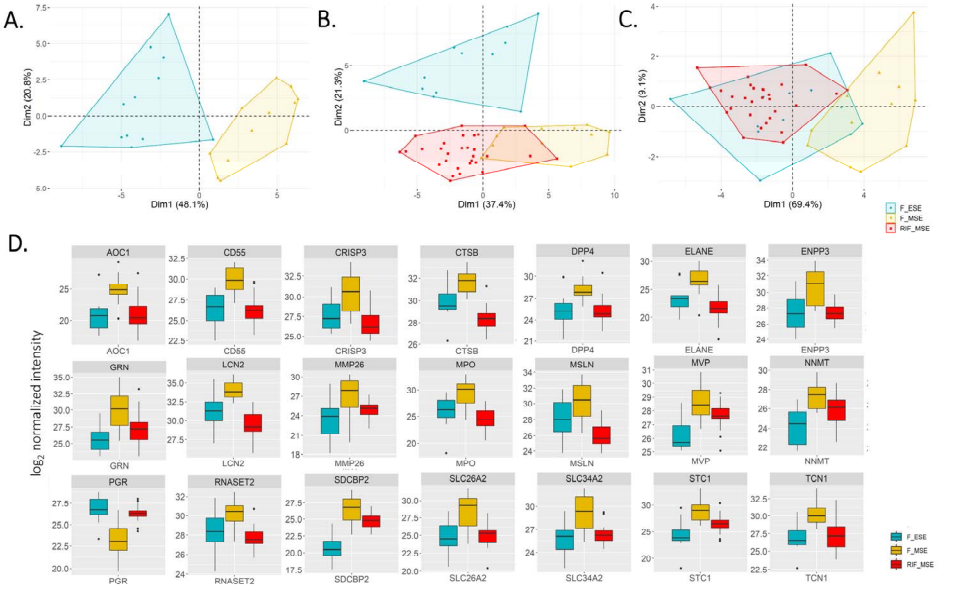
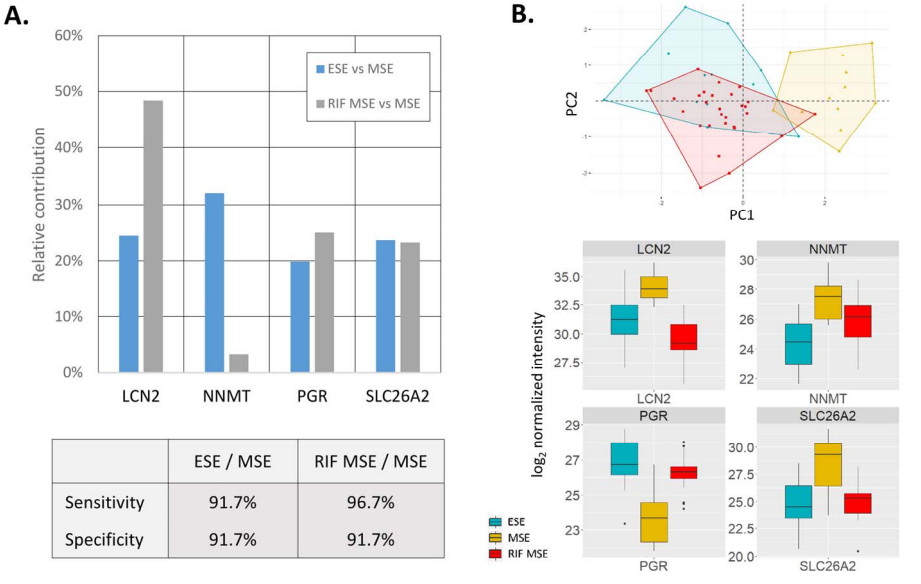


Figure 24. A. Validated ($p<0.05$) targeted proteins separate control ESE (*turquoise*) and MSE (*yellow*) groups into distinct PCA spaces illustrating their collective association with the respective cycle phases. **B.** PCA analysis including RIF MSE samples (*red*). **C.** Proteins highly significantly ($p<0.005$) different between control MSE and RIF MSE group, shifting RIF MSE to more closely overlap with the control ESE than control MSE samples. **D.** Proteins indicative of potential WOI displacement in women with RIF. The y-axis in the boxplots denotes normalized and log_2-transfromed summed peptide intensities of the respective proteins. To visualize group separation in the PCA the convex areas of sample groups have been color-coded.

5.2.6 Uterine fluid panel consisting of four proteins enables uterine receptivity monitoring with high sensitivity and specificity (Study II)

One of our main interests was whether uterine fluid proteins could be applied to monitor endometrial maturation and receptivity and how many proteins would be needed to provide sufficiently high sensitivity and specificity without rendering

the panel technically too complex and expensive for the potential development into an immunoassay. To that end, using the set of 21 validated and RIF displaced proteins, we combinatorically tested different three (1,330 combinations) and four (5,985 combinations) protein panels and determined how they distinct receptive samples from pre-receptive samples or RIF affected samples. Using Random Forest machine learning, a panel consisting of PGR, NNMT, SLC26A2 and LCN2 was estimated to provide optimal results, enabling distinguishing of control MSE from ESE with both sensitivity and specificity of 91.7% and control MSE from RIF MSE with sensitivity of 96.6% and specificity of 91.7% (Figure 25A). All these four markers had RIF MSE levels closer to control ESE than to control MSE levels (Figure 25B), thus potentially indicating that our RIF cohort suffers from a displaced WOI. Positive and negative predictive values were not determined, as it is difficult to estimate what the exact prevalence of RIF is in the general population due to varied definitions for RIF (Bashiri et al., 2018; Laufer and Simon, 2012).



	ESE / MSE	RIF MSE / MSE
Sensitivity	91.7%	96.7%
Specificity	91.7%	91.7%

Figure 25. Panel consisting of uterine fluid proteins PGR, NNMT, SLC26A2 and LCN2 provides high specificity and sensitivity for separating MSE samples from ESE samples and RIF MSE from control MSE. **A.** Relative contribution of each protein in Random Forest classification models and the estimated sensitivity/specificity of the panel. **B.** Levels of the panel proteins in RIF MSE group are more similar to control ESE than to control MSE, which are also reflected in the PCA plot, Individual boxplots of the proteins are shown below.

6. DISCUSSION

6.1 Shared molecular properties between endometriotic cells and those of tumorigenic or stem cell origin (Study I)

The principal finding of our endometriosis stromal cell study highlighted extensive changes in the protein expression of enzymes involved in energy metabolism indicative of a pseudohypoxic state of ectopic ESCs (Figures 12 and 13) which we later confirmed by directly measuring reduced oxygen consumption in ecESCs (Figure 15). Such increased glycolytic and lowered oxidative energy production under normoxic conditions has been a long-known phenomenon in tumor cells, known as the Warburg effect (Vander Heiden et al., 2009; Warburg, 1956). In addition to our results, several other studies have reported the potential significance of HIF1A, hypoxia and the Warburg effect in endometriosis (Kato et al., 2012; Wu et al., 2007; Wu et al., 2019; Young et al., 2016; Young et al., 2014).

Hypoxia-adaptive gene expression is regulated by the hypoxia-inducible factors (HIFs), which are made up of actively regulated α-subunits (HIF1A, HIF2A, HIF3A) and a constitutively expressed β-subunit, by binding to the hypoxia-responsive element (Mohlin et al., 2017). Under normoxia, HIF1A (the main α-subunit) is expressed, but quickly degraded by hydroxylation of its key proline residues by prolyl hydroxylase domain (PHD) proteins. This leads to the subsequent binding of the von Hippel-Lindau tumor suppressor protein (VHL) and proteasomal degradation of HIF1A (Maxwell et al., 1999). This critical hydroxylation is reduced under hypoxia, which then leads to the stabilization of the protein and enables it to exert its gene regulatory functions – affecting over 1,000 target genes (Hayashi et al., 2019) involved in neoangiogenesis, cell proliferation, invasion, survival and apoptosis among others.

Oxygen levels are not the only way HIFAs are regulated and multiple oxygen-independent mechanism have been described (Masoud and Li, 2015) – some of which may have relevance for endometriosis. Multiple growth factors appear to upregulate transcription and translation of HIF1A and for endometriosis the role of TGF-β1 through the down-regulation of inhibitor of DNA-binding protein 2 (ID2) has been pointed out (Young et al., 2016), as well as interleukin-6 (IL-6) mediated upregulation of pSTAT3 (phosphorylated signal transducer and activator of transcription 3), which stabilizes HIF1A (Kim et al., 2015). Another mechanism by which HIF1A can be stabilized under normoxia is through the increased production of reactive oxygen species (ROS) by inactivating oxidization of the Fe^{2+} cofactor of PHD (Gerald et al., 2004). ROS is known to be increased in endometriotic cells and the peritoneal environment of endometriosis patients (Scutiero et al., 2017). We also observed increased normoxic HIF1A mRNA in ecESCs, nevertheless, under primary cell culture conditions no TGF-β1 nor IL-6 stimulation was used. It is therefore plausible that higher ROS plays a contributory role in the induction of the Warburg effect in ecESCs, but remains to be proven.

There are multiple potential pathogenetic consequences for endometriotic cells from the anaerobic metabolism. Our results showed that LDHA (mainly converts pyruvate to lactate) is upregulated by ~250%, while LDHB (mainly converts lactate to pyruvate) is downregulated in endometriotic cells. This signifies that peritoneal ecESCs produce net elevation in lactate levels, which is also corroborated by observations of increased lactate in the peritoneal cavity of endometriosis patients (Young et al., 2014). Lactate is well known to promote migration, invasiveness and immune evasion of tumor cells (Hirschhaeuser et al., 2011) – properties which have also been ascribed to endometriotic cells (Aznaurova et al., 2014). There is clearly a reason why the immune system tends to ignore ectopic growth of endometrial cells. In addition to altered cytokine levels (TGF-β1, IL-6, IL-10, IL-15, IL-17, IL-33, TNF-α), increased local acidity through elevated lactic acid may at least partially provide the answer by promoting immunotolerance (Sun et al., 2017; Symons et al., 2018). Attenuated and changed activity of immune cells in response to altered microenvironment around the endometriotic lesions is now considered to be an important aspect in the persistence of ectopic lesions (Symons et al., 2018). Albeit, it is less clear how much does it contribute to the initial development of the lesions and whether the altered microenvironment precedes or follows the development of endometriosis.

Warburg effect is also exhibited by stem cells and gradual differentiation of cells is accompanied by gradual shift in energy metabolism from anabolic to aerobic (Xu et al., 2013). Increased pseudohypoxia and glycolytic activity is an inherent feature of MSCs (Palomaki et al., 2013), that also perform a critical function in regenerating the endometrium after each menstrual shedding and are thought to play a role in endometriosis (Djokovic and Calhaz-Jorge, 2015). In fact, during menstruation endometriosis patients compared to healthy controls have increased shedding of the basalis layer of the endometrium which harbors MSCs (Leyendecker et al., 2002). Therefore, it is possible that the pseudohypoxic differences we see in our results between ESCs from lesions and the endometrium are the result of different level of 'stemness' of the ESCs, *i.e.* ectopic lesions are established by the more stem cell-like stromal cells compared to stromal cells biopsied from the eutopic endometrium.

In addition to metabolic changes, we also measured significant changes in proteins related to cell survival, proliferation, invasiveness and cellular adhesiveness in ecESCs. Primary ecESCs mainly appear to have downregulated pro-apoptotic proteins, while anti-apoptotic proteins are mostly upregulated (Table 6). Number of the tumor suppressors that our data cover have also been found to be reduced in endometriotic lesions by other studies, such as BAX (Cho et al., 2018), CASP1 (Braun et al., 2007) and PAMR1 (Kobayashi et al., 2012), while nuclear SMAD4 was found to be unchanged in the eutopic endometriotic endometrium (Dela Cruz et al., 2015) and loss of heterozygosity of CDKN2A has been associated with endometriosis (Goumenou et al., 2000). The expression of AIFM2, CASP7 and DAPK3 have not been reported in endometriosis to our knowledge. Among the pro-survival proteins only the proto-oncogenic CTNNB1 has been implicated in ectopic endometriotic cysts (Pazhohan et al., 2018),

whereas no data exist for CTNNA1/2 and TPD52L1, while HRAS has been only investigated for mutations with negative findings (Vestergaard et al., 2011). It is noteworthy that some changes appear to suggest anti-proliferative adaptions instead, exemplified by CDKNA2A and API5, perhaps as a counter-response to the aforementioned pro-proliferation expressional changes. Nevertheless, most of the results are in concordance with other studies that have emphasized increase in proliferation and reduced apoptosis of endometriotic cells.

When looking at the highest effect sizes among upregulated proteins in ecESCs, a number of pro-invasiveness related proteins top the list: SERPINE2 (~31-fold compared to control euESCs) and NNMT (~9-fold). Both of these proteins carry prominent roles in cellular invasiveness and migration, but also in normal functioning of the endometrium. SERPINE2 has been implicated in cellular invasiveness in multiple studies (Buchholz et al., 2003; Gao et al., 2008; Nagahara et al., 2010; Selzer-Plon et al., 2009; Wang et al., 2015), and is expressed in the endometrium during the WOI where it has been suggested to enable tissue remodeling prior to implantation (Lee et al., 2011b). NNMT was recently demonstrated to be the master regulator in the transformation of cancer-associated stromal fibroblasts from *in situ* to a metastatic phenotype and affecting the expression of thousands of genes (Eckert et al., 2019). NNMT has also been shown to play important role in proliferation and invasiveness in other cell types (Hah et al., 2019; Tang et al., 2011), and it has been shown to be upregulated in endometriotic lesion tissues (Eyster et al., 2007). Interestingly, NNMT showed a trend in our data for elevated levels also in endometriotic euESCs which may implicate its more important role in the overall endometriosis pathogenesis. Moreover, NNMT was also highly upregulated during MSE in our uterine fluid study, which may, similarly to SERPINE2, suggest its role in implantation in the normal functioning of the endometrium, but which may endow undesirable properties to ESCs in the development of endometriotic lesions. Other upregulated proteins in ecESCs involved in cellular adhesion and motility are presented on Figure 14A, overall, supporting that ecESCs are characterized by adhesive and invasive properties.

The main limitation of studying primary ESCs is related to the use of cultured cells. Culturing the cells after isolation from patient samples may theoretically alter the phenotype that these cells express in response to the *in vivo* micro-environment, or culturing may introduce alterations that are only present *in vitro*. Nevertheless, it was shown by a recent study that culturing endometriotic cells does not really alter their phenotypes and such cells retain their characteristic molecular markers (Bouquet De Joliniere et al., 2014). It is also desirable to study only a specific cell population compared to whole tissue, which is made up from multiple cell types and may not have a reproducible profile from biopsy to biopsy. This problem that arises from tissue heterogeneity in endometriosis studies has been reviewed and discussed by our group elsewhere (Saare et al., 2017). Another limitation of our study is the usage of ecESCs only from peritoneal implants, as the observed changes may not be directly carried over to ovarian endometriomas. Finally, our samples were all collected during the secretory phase, which may

limit the gained insight only to the secretory phase of the menstrual cycle. Peritoneal lactate differs more distinctly in the secretory phase between endometriosis patients and controls and less so in the proliferative phase (Young et al., 2014), suggesting that the induction of metabolic effects may also be cyclical if they are driven by hormonal signaling.

6.2 Proteomics avenues for the diagnostics of endometriosis (Study I)

Blood-based biomarkers for the non-invasive diagnostics of endometriosis have been a long sought-out goal for multiple previous studies with no definitively useful markers yet found (Nisenblat et al., 2016). We decided to approach the blood plasma proteomic discovery of endometriosis markers with an upstream immunodepletion and peptide fractionation – an approach which has not yet been attempted to our knowledge. However, a drawback for such a workflow is the laborious processing and prolonged instrumental analysis time of samples. Therefore, to include as many patients and controls as available, we used experimental design based on pooled samples. However, we did not detect any meaningful differences between patients and controls nor did subgrouping reveal any proteins different between groups. The only protein for which we detected a trend for significance was LPA, which appears to be decreased in the luteal versus follicular phase plasma of controls, whereas no change is observable for endometriosis. It is noteworthy, that LPA and its associated lipoprotein particle, lipoprotein(a), concentrations have been reported to be elevated in women with endometriosis (Crook et al., 1997). Nevertheless, we did not consider this level of evidence of further interest nor specific enough for endometriosis, as apolipoproteins are altered in other conditions (Saleheen et al., 2017).

Recent blood discovery studies for endometriosis similarly did not find neither specific nor sensitive markers or marker sets for further diagnostic development. A rigorous immunological plasma study of multiple cytokines in a large and well-defined cohort showed that plasma cytokines do not enable differentiation of patients from controls (Knific et al., 2019). A shotgun proteomics study with pooling and immunodepletion did report differing proteins in endometriosis versus control serum samples, but no correction for multiple testing was carried out in this study, nor were the selected markers significant in a later follow-up validation stage (Irungu et al., 2019). Similar problems were apparent also in another proteomics study, *i.e.* no markers were reported with rigorous statistics (Manousopoulou et al., 2019). Any p-values from (prote)omics studies with hundreds or thousands of simultaneous tests need to be corrected for false discovery that arises in these situations (Krzywinski and Altman, 2014), especially when reporting candidates for diagnostic purposes that need clear and consistent separation for high sensitivity/specificity. Despite technological advances in LC/MS/MS technology, current MS-based plasma proteomics approaches may

still not yet be ripe for detecting the ultra-low-abundant proteins that leak from diseased tissues. Foremost, due to the 10^{10} concentration range problem presented by blood plasma proteins (Geyer et al., 2017). Therefore, in the arrival of technological improvements that address concentration dynamic range related issues in blood plasma, studies on endometriosis-specific proteins may need to be revisited.

6.3 Suitability of uterine fluid proteins for endometrial receptivity monitoring (Study II, III and IV)

It is estimated that about two thirds of implantation failures are caused by defects in endometrial receptivity and the remaining one third by embryo-side problems (Craciunas et al., 2019). Thus, minimally invasive monitoring of endometrial receptivity could help to improve IVF success rates, facilitate identification of endometrial factor RIF patients and reduce costs for the healthcare system by avoiding futile procedures. In our proof-of-concept study, we show that uterine fluid proteins can provide useful information on endometrial receptivity, while remaining minimally invasive and useable within the same cycle as the fluid sampling.

The protein composition of uterine fluid is complex with many high abundant proteins dominating the proteome – quite similarly to blood plasma. This similarity may suggest that there is significant transudation of proteins from plasma, although, these proteins have been shown to be expressed also in endometrial glandular epithelial cells (Hannan et al., 2010). Nevertheless, their concentrations compared to the rest of the proteome are not as extreme as in blood plasma, and after moderate fractionation, we were able to identify ~3,200 proteins in the fluid from healthy fertile controls. About one tenth of them significantly change from ESE to MSE coinciding with the opening of the WOI. Our bioinformatics analyses indicated that most of the proteins are annotated as extracellular, extracellular matrix-associated, plasma membrane, exosomal or secreted, while there is also significant amount of proteins known to be intracellular or cytosolic – although, many of the identified cytosolic proteins are known to be partitioned into extracellular vesicles as well. In addition, we detected multiple known exosomal markers (*e.g.* CD9, CD81, CD63) (Kowal et al., 2016), which would indicate that there is a significant amount of extracellular vesicles in uterine lavage. Presence of extracellular vesicles in uterine cavity has also been reported by others (Ng et al., 2013), and animal studies have suggested that they play a role in embryo-endometrium cross-talk (Zhang et al., 2017). Nevertheless, it cannot be ruled out that uterine fluid also contains material from damaged or lysed endometrial cells to some extent.

We found that many differentially abundant fluid proteins between ESE and MSE have previously been reported in the context of endometrial receptivity. The mRNA levels of 20 of these proteins are also measured in the widely used ERA® test. It is noteworthy that majority (18/20) of the shared ERA® genes show up- or

downregulation in the same direction as their respective uterine fluid proteins. This shows that the significantly changing uterine fluid proteins can reflect tissue gene expression. Nevertheless, the overall overlap of genes used in ERA® and proteins quantifiable in the fluid was only 54/238. Thus, 34 of the transcriptionally different genes in endometrial tissue do not show meaningful differences on the fluid protein level, or their mRNA/protein correlation is inherently low in the tissue. In dynamic states, such as when the endometrium transitions from the pre-receptive to the receptive state, mRNA/protein correlation can be low compared to steady states where there is more correlation (Liu et al., 2016). This can be either due to time delay between protein synthesis and transcription, post-transcriptional regulation, variable protein stability, location-dependent synthesis and/or trafficking of proteins (Liu et al., 2016). For ENPP3 we saw that even though it is transcribed in endometrial stromal cells, there is almost no detectable protein in the stroma, and the protein is predominantly present only in the apical region of the glandular cells from where it also likely makes it to the uterine fluid. Throughout various time points of the menstrual cycle ENPP3 mRNA and protein showed much better correlation in glandular cells, also mirroring the dynamics we observed in uterine fluid. Thus, the correlation between mRNA and protein can also depend on the particular cell type observed in the tissue. For STC1 we did not study cell type-specific expression of its mRNA, but STC1 protein was present in all cell types of the endometrium. Dynamics of the overall tissue mRNA of STC1 was poorly correlated with STC1 protein in the uterine fluid, suggesting that the eventual levels in the fluid are determined by other processes than mRNA transcription.

Among the significantly changing uterine fluid proteins found in our discovery data, we were able to validate 38 out of 45 proteins using targeted MS and samples from independent controls. There may be several reasons why seven of the 45 candidates were not validated. The validation samples were collected from three different clinics instead of one, as in the discovery part of the study. Thus, there could be more technical variability in the data, although, the validation cohort better represents the results that will be measured in an inter-clinical real-life context, where it is desirable to have only robust biomarkers. Finally, failure to replicate some of the effects observed in small discovery studies in larger ones may come down to the higher biological variability that larger populations have.

From technical side of uterine fluid analysis, it is important to emphasize that we observed variable volumes of the lavage fluid that was collected in the clinics. As mentioned before, even though the volume of the uterine fluid somewhat fluctuates throughout the menstrual cycle, there were notable intra-cycle phase differences observed as well, that appeared to be related to how much lavage fluid was aspirated after flushing the uterus. These volume-related factors can lead to different measured concentrations of proteins even though the absolute quantities in the uterus do not change. To capture changes in total amounts, we used reference normalization for which EEF1A1 showed most stable levels between ESE and MSE. Based on the overlap of results for several genes between our study and previous studies obtained with biopsies, we conclude that this approach counteracts

these factors when comparing ESE and MSE time points. Overall, we considered the remaining 38 proteins as validated and sufficiently reproducible to merit further developments into an assay.

When estimating the sensitivity and specificity of the validated proteins, using machine learning we arrived at a four-protein panel (PGR+NNMT+SLC26A2+ LCN2) which showed high sensitivity and specificity of 91.7% for estimating whether the endometrium is pre-receptive or receptive. Such a four-protein panel is also conveniently smaller than the entire fluid protein complement, which is an important consideration if the assay is to be transferred onto an ELISA-based platform. For classifying subjects as 'RIF' when analyzing RIF samples that are collected 7–9 days after the LH-surge, the panel demonstrated sensitivity of 96.6% and specificity of 91.7%. In a theoretical future clinical use, a 'RIF' result by this panel would direct the patient into further sampling of the fluid outside the previous LH-test time point to determine whether a 'receptive' status is achieved (subject has displaced WOI) or not (subject has disrupted WOI). The latter case may then signify that in such a subject attempting IVF-ET without other interventions is futile.

When attempting to compare the performance of our panel to that of the ERA® test, the latter has been reported to give sensitivity and specificity of 88.6% and 99.8%, respectively, for endometrial dating. Differentiation of non-pathological/ pathological (pathology defined as ≥5 implantation failures or with hydrosalpinx) MSE was achieved with a sensitivity of 99.5% and specificity of 15.7% (Diaz-Gimeno et al., 2011a). However, in the current stage the performance characteristics of these two approaches cannot be accurately compared head-to-head, as our and ERA® control cohorts consisted of 17 and 88 individuals, respectively, while for altered receptivity detection our study had 29 RIF and ERA® 7 mostly-RIF patients. The molecular signature of ERA® has, however, been applied to RIF patients in a number of follow-up studies with varying levels of success for improving implantation rates in ERA 'non-receptive' RIF patients undergoing personalized IVF and ET (Patel et al., 2019; Ruiz-Alonso et al., 2013).

The performance of our predictors may change when applied to a larger population. Nevertheless, our results suggest that during MSE, uterine fluid proteins might be superior in determining whether a patient suffers from an endometrial-factor RIF or not, while performance in endometrial dating is similar or slightly less than ERA®. Overall, uterine fluid proteins show promising results for improving minimally invasive endometrial receptivity assessment both in healthy patients and in women with RIF of unknown origin.

6.4 Role of endometrial factors in women with recurrent implantation failure of unknown origin reflected in uterine fluid (Study II)

We found that 21 uterine fluid proteins out of 38 validated receptivity-associated proteins in our RIF MSE cohort had the same levels as in control ESE samples. The above-mentioned four-protein panel, consisting of PGR, NNMT, SLC26A2 and LCN2 enabled high discrimination of RIF MSE from control MSE. Of this panel, only LCN2 has not previously been implicated in endometrial receptivity, whereas PGR, NNMT and SLC26A2 have been reported in the context of endometrial receptivity (Chan et al., 2013; Qiao et al., 2008).

Persistent elevation of the progesterone receptor or PGR expression in endometrial epithelial cells was initially shown to be associated with luteal phase defects (Lessey et al., 1996) – a condition where there is low secretion of progesterone from the ovaries or diminished responsiveness of the endometrium to progesterone. High PGR expression in the glandular epithelium is considered to be a hallmark of progesterone resistance – a prominent feature of many infertility associated conditions (Fox et al., 2016). Similarly, in our study, RIF MSE and control ESE had higher levels of PGR than control MSE uterine fluid samples. The expression of nicotinamide N-methyltransferase or NNMT in the endometrium is also regulated by progesterone, but also by estrogen, and during the WOI it is normally downregulated in the endometrium (Tapia-Pizarro et al., 2014). NNMT is a multifaceted enzyme that catalyzes the metabolism of nicotinamide and various xenobiotics. As discussed in the context of our endometriosis study, it influences the expression of multiple of genes, which are involved in proliferation and cellular migration. Similarly to NNMT, the expression of sulfate transporter or SLC26A2 is under the control of progesterone (Dassen et al., 2007). Its main function is the transport of several different anions across lipid bilayers and its expression is attenuated during the WOI in women with PCOS (Qiao et al., 2008). Not much is known about the role of neutrophil gelatinase-associated lipocalin or LCN in human endometrial function, but the fertility of $LCN2^{-/-}$knockout mice is impaired (Berger et al., 2006).

Overall, the results of uterine fluid proteins clearly show that there are alterations in uterine functioning of women with RIF. Future studies should also address whether the uterine fluid panel addresses only RIFs that are caused by displaced gene expression or also RIF cases that have a more generally disrupted WOI.

7. CONCLUSIONS

Considering the findings of the studies presented in this thesis, following conclusions can be drawn:

1. Primary ectopic endometrial stromal cells are substantially different from their eutopic counterparts, as evidenced by pathway-wide changes in anaerobic and oxidative cellular metabolism. These changes are characterized by reduced oxidative metabolism and increased glycolysis in the presence of oxygen – a phenomenon known as the (pseudohypoxic) Warburg effect. The ectopic endometrial stromal cells also have higher levels of HIF1A and display reduced oxidative respiration compared to eutopic cells. In addition, ectopic endometrial stromal cells have upregulated proteins involved in cellular motility, adhesiveness and invasiveness, most notably proteins such as SERPINE2 and NNMT, which normally play a role in implantation and endometrial receptivity. Many known pro- (*e.g.* caspase-1/7, SMAD4, BAX and others) and anti-apoptotic (*e.g.* HRAS, CTNNA1, CTNNA2, CTNNB1) proteins are down- and upregulated, respectively, in primary ectopic endometriotic cells, which is in agreement with previous reports of reduced apoptotic potential of endometriotic cells.
2. Immunodepleted blood plasma proteome analyzed to a depth of ~1000 proteins does not enable separation of endometriosis patients from patients affected by other gynecologic conditions.
3. Proteins secreted to the uterine fluid make up a complex and dynamic proteome that significantly changes in transitioning from a pre-receptive to a receptive state during the endometrial secretory phase.
4. Endometrial tissue and cell-specific mRNA and protein expression may not correlate to a high degree, as evidenced by ENPP3 and STC1. *ENPP3* gene is transcribed in all cells of the endometrium, but its protein is expressed only in the apical glandular epithelium. *STC1* tissue mRNA expression throughout the menstrual cycle is more variable than its protein levels in uterine fluid.
5. Protein signature consisting of 21 receptivity-specific uterine fluid proteins suggests alteration of the window of implantation in women with recurrent implantation failure of unknown origin. A four-protein panel consisting of PGR+NNMT+SLC26A2+LCN2 enables high accuracy for endometrial receptivity detection with a specificity and sensitivity of 91.7%. The same panel discriminates RIF mid-secretory samples from non-RIF samples with a sensitivity and specificity of 96.6% and 91.7%, respectively.

8. SUMMARY IN ESTONIAN

Inimese endomeetriumi normaalne ja patoloogiline profiil proteoomika vaatevinklist

DNA ja RNA sekveneerimisel põhinevad oomika-meetodid on märkimisväärselt panustanud reproduktiivmeditsiini arengusse, aidates selgitada haiguste põhjuslikke tagamaid ning võimaldanud uusi viise nende haiguste diagnoosimiseks. Mass-spektromeetria (MS) tehnoloogiatel põhineva proteoomika panus on genoomika ja transkriptoomika kõrval olnud mõnevõrra tagasihoidlikum ning seda peamiselt valkude mitmekesisusega seotud tehniliste raskuste tõttu. Siiski on pärast viimase kümnendi MS-proteoomika progressiivset arengut rakuproteoomide terviklik määramine lõpuks teostatav. Valkude kvantitatiivne määramine ja iseloomustamine on oluline, kuivõrd proteoom on genoomi peamine funktsionaalne väljund, pealegi on näidatud, et transkriptide tase peegeldab valkude tegelikku ekspressiooni ainult osaliselt. Kaasaegse MS-proteoomika laialdasem rakendamine võimaldaks leida uusi lahendusi ka mitmetele reproduktiivmeditsiiniga seotud probleemidele, nagu haiguste molekulaarsete mehhanismide väljaselgitamine ja kehavälise viljastamise efektiivsuse tõstmine.

Reproduktiivmeditsiinis on jätkuvalt oluliseks teemaks endometrioosi tekkepõhjused. Endometrioos on sagedalt (~5–10% viljakas eas naisi) esinev günekoloogiline haigus, millega kaasneb emakaõõne limaskesta ehk endomeetriumi rakkude levik ja ellujäämine väljaspool emakat. Haigusega kaasnevad sageli krooniline valu, viljatus ja meeleoluhäireid, mis toovad kaasa patsientide elukvaliteedi märkimisväärse languse.. Endometrioosi täpne diagnoos määratakse laparoskoopilise operatsiooniga, mis omab kirurgilisest protseduurist lähtuvaid ohte ja on tervishoiusektorile kulukas. Kuna endometrioosi sümptomid kattuvad mitmete teiste haigustega ja siiani puuduvad diagnostikaks mitte-invasiivsed biomarkerid, võib diagnoosini jõudmine aega võtta aastaid pärast esimeste kaebuste tekkimist. Seetõttu võib endometrioosi kolderakkude ja patsientide vereproteoomide uurimine anda uusi vihjeid haiguse patogeneesi kohta ning välja pakkuda perspektiivseid biomarkereid endometrioosi diagnoosimiseks.

Kehaväline viljastamine ehk IVF on üha sagedamini teostatav protseduur ja seda eelkõige aina hilisemasse ikka nihkunud pereplaneerimise tõttu. IVF kui meditsiiniline protseduur on aga madala efektiivsusega, kuna ainult ~30% protseduuri läbinud naistest rasestub. Üheks sagedaseks põhjuseks on siiratud embrüote ebaõnnestunud implantatsioon, mis osade naiste korral osutub korduvaks ja otseselt mitteseletatavaks probleemiks. Seetõttu arvatakse, et neil IVF patsientidel võib esineda häireid endomeetriumi retseptiivsuse või loomuliku arenguga. Emakaõõne sekreedist retseptiivsusega seotud valguliste mustrite määramine võimaldaks efektiivsemalt tuvastada aega, millal konkreetsele patsiendile IVF embrüot siirata või tuvastada, kas protseduur on üldse edukalt teostatav. Taoline lähenemine vähendaks aja- ja materiaalset kulu, mis korduvalt ebaõnnestuvate protseduuridega paratamatult kaasneb.

Käesoleva väitekirja üheks eesmärgiks oli rakendada kaasaegse proteoomika meetodeid endometrioosipatsientide endomeetriumi ja kõhuõõne kollete rakkude uurimiseks ning vereplasmast haigusele spetsiifiliste markerite tuvastamiseks. Teine osa tööst käsitleb emakasekreedi proteoomikat, mille käigus uuriti, kas sekreedivalgud sobivad endomeetriumi retseptiivsuse vähe-invasiivseks määramiseks ja retseptiivsushäirete tuvastamiseks.

Uurimistöö täpsemad eesmärgid olid:

- Võrrelda endometrioosipatsientide kõhuõõne kolletest ja eutoopilisest endomeetriumist eraldatud stroomarakkude valgulisi profiile ja kõrvutada neid tervete naiste omaga, et tuvastada kolderakkudes toimunud patogeneetilisi protsesse.
- Tuvastada uusi potentsiaalseid endometrioosi-spetsiifilisi markereid vereplasma valkude seast.
- Iseloomustada endomeetriumi sekretoorse faasi sekreedi valgulist komplekti ja tuvastada sekreedi koostise muutused endomeetriumi üleminekul eelretseptiivsest retseptiivsesse faasi.
- Määrata endomeetriumi koe ja sekreedi geenieksпressiooni omavaheline kattuvus.
- Hinnata emakasekreedi valgupaneeli sensitiivsust ja spetsiifilisust endomeetriumi retseptiivsuse määramiseks ja selle häirete tuvastamiseks.

Materjalid ja meetodid:

Endometrioosi stroomarakkude proteoomika uuringuteks värvati laparoskoopilisele kirurgiale suunatud naisi, kellel diagnoositi endometrioos ning kellelt koguti endomeetriumi (n=5) ja kõhuõõne kollete (n=6) biopsiad. Kontrollideks värvati terveid ja viljakaid naisi (n=5). Biopsiatest eraldatud stroomarakke kultiveeriti 3–5 passaaži, paralleelselt valmistati SILAC aminohapetega valmistatud standard. Rakuproovidest eraldati valgud ning proovid segati kokku standardi valkudega, millele järgnes valkude proteolüüs trüpsiiniga. Saadud peptiidiproovid analüüsiti vedelikkromatograafia-tandem-massispektromeetria (LC/MS/MS) meetodiga ning valkude identifitseerimine ja kvantiteerimine teostati MaxQuant tarkvaraga. Statistiliseks võrdlemiseks kasutati ANOVA (valepositiivsete määr <0,05 pärast mitmese testimise korrektsiooni) ja Tukey-Kramer analüüsi ($p<0,05$). Oluliselt muutuvaid valke hinnati KEGG ja GO terminite rikastusanalüüsiga, kasutades selleks tarkvarakeskonda DAVID. Tulemuste valideerimiseks kasutati qRT-PCR meetodit ja respiromeetriat, võrreldes oksüdatiivse hingamise erinevusi eutoopiliste ja ektoopiliste rakkude vahel. Endometrioosi-spetsiifiliste markerite uurimiseks vereplasmast kasutati vereproove patsientidelt (n=119) ja kontrollidelt (n=53). Kontrollgrupi moodustasid naised, kes olid suunatud laparoskoopiale endometrioosile viitavate sümptomite tõttu, kuid kellel endometrioosi ei tuvastatud. Mõlema grupi proovidest moodustati segud (endometrioosi segusid n=24,

kontrolle n=12), seejärel eemaldati proovidest plasma 14 kõige kõrgema kontsentratsiooniga valku, kasutades selleks MARS-14 immuunoafiinsuskolonni. Järelejäänud valgud sadestati ja trüpsinolüüsiti. Peptiide fraktsioneeriti aluselise pööratud faasi kromatograafiaga ja tuvastati LC/MS/MS meetodil. Tulemusi analüüsiti MaxQuanti Perseus tarkvaraga.

Emakasekreedist diagnostilise valgupaneeli tuvastamiseks teostati esmalt proteoomika avastamisfaasi uuring, kasutades selleks tervetelt viljakatelt naistelt (n=6) pärit varajase ja kesksekretoorse faasi endomeetriumi sekreete. Valgud eelfraktsioneeriti SDS-PAGE meetodil kuueks fraktsiooniks ja trüpsinolüüsiti geelis peptiidideks. Valkude tuvastamine ja kvantiteerimine viidi läbi LC/MS/MS ja MaxQuant analüüsiga. Statistiliseks võrdlemiseks normaliseeriti valguintensiivsused ja erinevuse olulisus määrati paaris t-testiga, korrigeerides q-väärtuse meetodiga ($q<0,05$) mitmese testimise suhtes. Sekreedi valkude rakulist lokalisatsiooni analüüsiti GO-terminite rikastusega. Enimmuutuvate valkude (>5-kordne erinevus, n=45) põhjal koostati suunatud MS/MS meetod, mida rakendati uue rühma tervete kontrollide (n=12) ja korduva implantatsiooni-häirega (RIF, *recurrent implantation failure*) naiste (n=29) proovide mõõtmiseks. Juhumetsa (*random forest*) analüüsiga hinnati erinevaid 3- ja 4-valgumarkeriga paneele, et leida retseptiivsuse määramiseks tõhusaim markerite paneel.

QRT-PCR analüüsiga määrati endomeetriumi koes ja erinevates rakutüüpides *STC1* ja *ENPP3* geenieksprессioon. ENPP3 valgutaset ja rakuspetsiifilist ekspressiooni mõõdeti erinevates menstruaaltsükli faasides immunohistokeemia meetoditega. STC1 rakuspetsiifiline valgueksprессioon määrati kesksekretoorse endomeetriumi koeslaididelt.

Uurimistöö peamised tulemused ja järeldused:

LC/MS/MS analüüsi tulemus näitas, et endometrioosi patsientide ektoopilised stroomarakud on proteoomi tasemel eutoopilistest rakkudest oluliselt enam erinevad kui haigete ja tervete eutoopilised rakud omavahel (vastavalt ~1500 vs ~100 erineva ekspressiooniga valku). Ektoopilistes rakkudes on ulatuslikud ekspressioonilised muutused valkudes, mis seotud anaeroobse ja oksüdatiivse metabolismiga. Need muutused sarnanevad vähirakkudes kirjeldatud pseudohüpoksia-laadse Warburgi efektiga. Warburgi efekti korral kasutavad rakud normoksia tingimustes rohkem glükolüütilist ja vähem oksüdatiivset Metabolismi. Taoline kohastumus aitab suurendada biosünteetiliste prekursorite tootmist ning põhjustab fenotüüpset dediferentseerumist ja immuunseire nõrgenemist. Ektoopilistes rakkudes täheldasime samuti ligikaudu 2× kõrgemat HIF1A (hüpoksia vastuse induktor) mRNA taset ja vähenenud hapnikutarbimist eutoopiliste rakkudega võrreldes. Need tulemused kinnitavad, et ektoopilistes rakkudes on sõltumata hapniku olemasolust rakuline hüpoksia vastus suurenenud.

Lisaks Warburgi efektile täheldasime ektoopilistes rakkudes oluliselt kõrgemat rakkude liikumise, adhesiooni ja invasiivsusega seotud valkude ekspressiooni. Osa nendest valkudest on kõrgenenud tasemega ka endometrioosipatsientide eutoopilistes stroomarakkudes, mis viitab sellele, et endometrioosi korral leidub

juba endomeetriumis muutunud omadustega rakke. Kolderakkudes on tugevalt ülesreguleeritud sellised valgud nagu SERPINE2 (+3000%, $p=5\times10^{-7}$) ja NNMT (+900%, p=0,001). SERPINE2 ja NNMT täidavad eutoopilises endomeetriumis olulist füsioloogilist funktsiooni, osaledes embrüo implantatsioonis ja endomeetriumi retseptiivsuses. Kuidas need valgud osalevad endometrioosi patogeneesis on veel ebaselge, kuid tuumorites on nende kõrgenenud taset seostatud koeinvasiivsusega.

Eelnevate uuringutes on endometrioosi rakkudele omistatud kõrgenenud apoptoosiresistentsust ja aktiivsemat proliferatsiooni. Meie proteoomika andmed näitavad samuti, et kolderakkudes on mitmed pro- (kaspaas-1/7, SMAD4, BAX jt) ja anti-apoptootilised (HRAS, CTNNA1, CTNNA2, CTNNB1) valgud vastavalt alla- või ülesreguleeritud, mis toetab varasemaid täheldusi.

Endometrioosi patsientide veremarkerite uuringuks kasutasime enimesindatud plasmavalkude eemaldamisel põhinevat meetodit ja LC/MS/MS analüüsi, mis võimaldas meil tuvastada kokku 964 valku. Paraku statistiline võrdlus ei näidanud olulisi erinevusi patsientide ja kontrollide vereplasma valguprofiilide vahel. Üheks võimalikuks põhjuseks võib olla haiguse-spetsiifiliste valkude väga madal esindatus veres, mis jääb allapoole LC/MS/MS määramispiiri. Ka teised hiljutised endometrioosi vereplasma uuringud ei ole näidanud kliiniliselt kasutatavate haiguse-spetsiifiliste markerite leidumist.

Meie poolt teostatud emakasekreedi analüüsid näitavad, et sekreedivalgud (>3000 erinevat valku) moodustavad kompleksse ja dünaamilise proteoomi, mis muutub märkimisväärselt (n=367 valku, q<0,05) endomeetriumi üleminekul eelretseptiivsest staadiumist retseptiivsesse. Retseptiivsusega seotud valkude ekspressioonidünaamika kattuvuse hindamiseks sekreedi ja koe vahel uuriti ENPP3 ja STC1 geeni- ja valguekspressiooni endomeetriumirakkudes. ENPP3 mRNA ekspressioon tuvastati nii endomeetriumi strooma- kui ka epiteelirakkudes, kuid valk oli tuvastatav ainult näärmeepiteeli apikaalses osas ja emakasekreedis. Näärmeepiteeli ENPP3 valguekspressiooni muutumine menstruaaltsükli jooksul korreleerus emasekreedi LC/MS/MS mõõtmistega. STC1 valguekspressioon oli tuvastatav kõikides endomeetriumi rakutüüpides ja -sekreedis, kuid STC1 mRNA tase koes ei korreleerunud märkimisväärselt sekreedi valgutasemega. Need tulemused näitavad, et rakkude, koe ja sekreedi mRNA-valk tasemed kattuvad piiratud määral ning ei ole alati ennustatavad.

Emakasekreedi proteoomika tulemusi valideerisime suunatud MS/MS meetodiga, kasutades uusi kontrollproove ja kaasates mõõtmistesse ka RIF-patsiente. Ühtekokku valideerus 38 valku 45-st valitud markerist (p<0,05) ning 21 markeri korral täheldasime RIF-patsientide kesk-sekretoorse faasi proovides kontrollide varajase sekretoorse faasiga võrdväärseid tasemeid. Need tulemused viitavad potentsiaalsele nihkele RIF-patsientide endomeetriumi retseptiivsuse arengus, mis võib seletada, miks nendel naistel on embrüosiirdamised korduvalt ebaõnnestunud. Lisaks sellele tuvastasime neljast valgust koosneva paneeli (PGR+ NNMT+SLC26A2+LCN2), mis demonstreeris kõrget spetsiifilisust ja sensitiivsust nii endomeetriumi retseptiivsusakna (mõlemad 91,7%) kui ka RIF-staatuse (vastavalt 96,6% ja 91,7%) määramisel.

Kokkuvõtteks näitasime antud tööga, et kaasaegsete proteoomika meetodite rakendamine võimaldab saada uusi ja täpsemaid teadmisi molekulaarsete muutuste kohta, mis leiavad aset patogeneetiliste protsesside korral nagu endometrioos ja viljatus. Samuti võimaldab MS-proteoomika tuvastada perspektiivikaid biomarkereid, kuid väga keerulise maatriksiga proovide korral (vereplasma) on vaja veel edasisi tehnoloogilisi arenguid, et tõsta meetodite tundlikkust veelgi. Lisaks sellele eeldavad antud tööst saadud laiapõhjalised tulemused jätku-uuringuid ja optimeerimist enne nende potentsiaalset rakendamist kliinilises praktikas.

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1. **Heidi-Ingrid Maaroos**. The natural course of gastric ulcer in connection with chronic gastritis and *Helicobacter pylori*. Tartu, 1991.
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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.
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