Integrated eutopic endometrium and non-depleted serum quantitative proteomic analysis identifies candidate serological markers of endometriosis

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Abstract

Background: Endometriosis affects about 4% of women in the reproductive age and is associated with subfertility. The aim of the present study was to examine the quantitative proteomic profile of eutopic endometrium and serum from women with endometriosis compared to controls in order to identify candidate disease-specific serological markers.

Methods: Eutopic endometrium and serum from patients with endometriosis (n=8 for tissue and n=4 for serum) was respectively compared to endometrium and serum from females without endometriosis (n=8 for tissue and n=4 for serum) using a shotgun quantitative proteomics method. All study participants were at the proliferative phase of their menstrual cycle.

Results: At the tissue and serum level, 1,214 and 404 proteins were differentially expressed (DEPs) in eutopic endometrium and serum respectively of women with endometriosis vs. control. Gene ontology analysis showed that terms related to immune response | inflammation, cell adhesion | migration and blood coagulation were significantly enriched in the DEPs of eutopic endometrium as well as serum. Twenty-one DEPs had the same trend of differential expression in both matrices and can be further examined as potential disease- and tissue-specific serological markers of endometriosis.

Conclusions: The present in-depth proteomic profiling of eutopic endometrium and serum from women with endometriosis identified promising serological markers that can be further validated in larger cohorts for the minimally invasive diagnosis of endometriosis.

Introduction

Endometriosis is a gynaecological condition in which endometrial glands and stroma is implanted outside the uterine cavity, usually on the ovaries, Fallopian tubes and surrounding tissue within the peritoneal cavity (1). Endometriosis affects approximately 3 to 4% of women in the reproductive age (2) and the most common symptoms include pelvic pain, especially during menstruation, and subfertility (3).

The exact pathophysiology of endometriosis, related to infertility is still unknown. Endometriosis can be detrimental to fertility directly by distorting tubo-ovarian anatomy (4), or indirectly by invoking inflammatory (5) and oxidative damage (6,7) on the oocytes resulting in poorer quality oocytes. A non-invasive method of diagnosis is not available and currently, endometriosis can be only definitively diagnosed through laparoscopic surgery. Transvaginal sonography (TVS), as described in the consensus statement of the International Deep Endometriosis Analysis (IDEA) group, can also be used as a first-line imaging technique in order to examine women with suspected endometriosis (8).

Endometriosis persists in both the proliferative and secretory phases of the menstrual cycle. Rai *et al.* (9) reported an altered endometrial proteomic profile between proliferative and secretory/luteal phases of the menstrual cycle. Previous biomarker discovery studies have focused primarily on the secretory phase of the menstrual cycle, at the time where there is a significant level of protein turnover, modification and regeneration. The secretory or luteal phase can vary in individuals and in the context of fertility and implantation, the 'luteal phase defect' (10), coupled with the recent evidence around the non-specific timeframe of the 'implantation window' within the secretory phase of the menstrual cycle (11), means that the proteins related to the secretory phase are much more heterogeneous and may inadvertently conceal the discovery of non-menstrual cycle related endometriosis specific markers. For these reasons, in the present study, we focused on the proliferative phase for both controls and patients with endometriosis.

Non-targeted global proteomics, supported by recent technological advances in mass spectrometry, is gradually becoming an indispensable analytical tool in clinical research since

the unbiased protein expression profiling of tissue or serum/plasma can provide novel endophenotypic insight for a given pathophysiological state with unsurpassed analytical confidence. Such a strategy also provides great promise in the detection of novel diagnostic, prognostic and therapeutic targets that can eventually influence clinical practice (12-15).

There is a limited number of studies that have examined the global proteomic portrait of eutopic endometrium in women with endometriosis (16-18), and the serum/plasma proteomic profile of endometriosis patients (19-21) in order to identify tissue or blood level biomarkers for the diagnosis of endometriosis. However, the integrated quantitative global proteomic analysis of eutopic endometrium and non-depleted serum samples from women with endometriosis for the identification of candidate tissue- and disease-specific biomarkers using isobaric tags and state-of-the-art ultra-high precision LC-MS based methods has not been reported to date.

The aim of the present study was to apply an in-depth quantitative proteomics methodology in combination with comprehensive bioinformatics analysis to eutopic endometrium and serum from women with endometriosis during the proliferative phase of the menstrual cycle compared to healthy controls in order to identify potential serological markers for the minimally invasive diagnosis of endometriosis. An overview of the study workflow is presented in **Figure 1**.

Materials and Methods

Data recording, sample collection and tissue storage in this study were performed according to the World Endometriosis Research Foundation (WERF) Endometriosis Phenome and Biobanking Harmonisation Project (EPHect) (22-24). This study has institutional and regional review board approval by the University Hospital Southampton (RHMO&G160) and Hampshire B ethical committees (MREC08/ HO502/162).

Inclusion and exclusion criteria

Women in the endometriosis group had a laparoscopic diagnosis of endometriosis (laparoscopy or laparotomy) with the disease stage documented according to the ASRM classification [Stage I: minimal; Stage II: mild; Stage III: moderate; Stage IV: severe] (25, 26). Women undertaking endometrial biopsy had transvaginal ultrasonography or hysteroscopic inspection of their uterine cavity and this did not reveal any endometrial pathology. Patients with pelvic inflammatory disease were excluded from the study. The control group consisted of women with no endometriosis as diagnosed by a negative laparoscopy. Since the endometrial proteomic profile may vary in the different phases of the menstrual cycle, all participants (patients with endometriosis and healthy controls) were consistently at the non-menstruating proliferative phase of the menstrual cycle. Subfertility was defined as trying to conceive for more than 1 year without a successful outcome, while having regular sexual intercourse and not using any contraceptive methods.

Women were excluded from the study if they were age 45 years old and above, at the secretory phase of the menstrual cycle (15-28 day of menstrual cycle), on hormonal treatment within three months prior to the procedure, had a BMI of more than 30, or a current smoker. A systematic review and meta-analysis showed no association between smoking status and the development of endometriosis (27). However, smokers were excluded from our study because smoking has been shown to alter the blood plasma/serum proteomic profile (28). Due to the small number of subjects included in the present study we would be unable to correct for this potential confounder.

We selected women with regular cycles in order to more accurately define the proliferative phase. We included women with a history of regular menstrual cycles, and confirmed their stage of menstrual phase by their retrospective last menstrual date and the prospective date of menstruation. This may mean we excluded women with endometriosis and irregular cycles, but as menstrual cycle regularity has not been found to be significantly associated with endometriosis (29), we do not expect this inclusion criterion to significantly confound our results.

Patient recruitment

This study was performed at the Princess Anne Hospital, Southampton where suitable candidates were given an information sheet outlining the study and signed a consent form. Patients were grouped into those with endometriosis and those without (control) in accordance with the findings during laparoscopy. The findings of the laparoscopy were documented in the proforma. Whenever possible photographic evidence was obtained.

Endometrial tissue collection

Endometrial tissue was collected using endometrium sampler (Endocell®, Wallach, USA). Sample collection was performed before any uterine manipulation or procedure. Endometrial tissues that were suctioned in the tube were collected into individual falcon tubes containing normal saline. The procedure was repeated at least twice or until an adequate tissue sample was obtained.

Processing of endometrium sample and storage

The collected tissue samples were processed up to 4 hours from the collection. Tissues were transferred into a petri dish and were gently teased apart with a tissue forceps and then washed repeatedly with Phosphate Buffered Saline (PBS) to remove any blood. Healthy tissues that were free from blood were cut into smaller pieces (approximately 15mm in length) using a pair of tissue scissors. The processed tissues were then transferred into at least 3 separate Cryovials (Greiner, UK). These vials were snap frozen in -80 °C freezer. Endometrium was transported on solid carbon dioxide (dry ice) inside a polystyrene box.

Quantitative proteomics sample processing

Two independent multiplex experiments were performed to include specimens from 16 subjects (n=8 controls; n=8 females with endometriosis). Specimens were dissolved in 0.5 M triethylammonium bicarbonate, 0.05% sodium dodecyl sulphate and subjected to pulsed probe sonication (Misonix, Farmingdale, NY, USA). Lysates were centrifuged (16,000 g, 10

min, 4°C) and supernatants were measured for protein content using infrared spectroscopy (Merck Millipore, Darmstadt, Germany). Lysates were then reduced, alkylated and subjected to trypsin proteolysis. Peptides were labelled using the eight-plex isobaric Tag for Relative and Absolute Quantitation (iTRAQ) reagent kit (Label assignment, Experiment A: 113=control 1, 114=control 2, 115= control 3, 116= control 4, 117= endometriosis patient 1, 118= endometriosis patient 2, 119= endometriosis patient 3, 121= endometriosis patient 4; Experiment B: 113=control 5, 114=control 6, 115= control 7, 116= control 8, 117= endometriosis patient 5, 118= endometriosis patient 6, 119= endometriosis patient 7, 121= endometriosis patient 8) and analysed using multi-dimensional liquid chromatography and tandem mass spectrometry as reported previously by the authors (30-34).

Serum procurement and proteomic analysis

The procurement and handling of sera was in accordance with the recommendations of the Standard Operating Procedure Integration Working Group (SOPIWG) as adopted by the author's method (35). One eight-plex serum proteomics experiment was performed (n=4 controls; n=4 patients with endometriosis). Serum specimens were freshly thawed and vortexed for 2 minutes. For each participant, 100uL of unprocessed serum were mixed with 400uL 6M Guanidine Hydrochloride and subjected to global quantitative serum proteomic analysis using our reported depletion-free methodology (12-14). In summary, high-performance Size Exclusion Chromatography using three serially connected Waters KW-804 columns at 0.75 ml/min flow rate and 30°C was used to separate the proteins based on their molecular weight differences. The separated low-molecular weight protein segments (molecular weight cutoff 3 kDa) were dialysis purified and lyophilized to dryness. One-hundred µg of protein from each sample was subjected to trypsin proteolysis and the peptides were chemically labelled using the eight-plex iTRAQ reagent kit (Label assignment, 113=control 9, 114=control 10, 115= control 11, 116= control 12, 117= endometriosis patient 9, 118= endometriosis patient 10, 119= endometriosis patient 11, 121= endometriosis patient 12), pooled, and offline fractionated with high pH C_4 reverse phase chromatography. Each fraction

was analysed using ultra-high performance low pH C_{18} nano-liquid chromatography hyphenated with high-resolution tandem mass spectrometry using the FT-Orbitrap Elite platform.

Database searching

Unprocessed raw files were submitted to Proteome Discoverer 1.4 for target decoy search against the UniProtKB homo sapiens database comprised of 20,159 entries (release date January 2015), allowing for up to two missed cleavages, a precursor mass tolerance of 10ppm, a minimum peptide length of six and a maximum of two variable (one equal) modifications of; iTRAQ 8-plex (Y), oxidation (M), deamidation (N, Q), or phosphorylation (S, T, Y). Methylthio (C) and iTRAQ (K, Y and N-terminus) were set as fixed modifications. FDR at the peptide level was set at <0.05. Percent co-isolation excluding peptides from quantitation was set at 50. Reporter ion ratios from unique peptides only were taken into consideration for the quantitation of the respective protein. The iTRAQ ratios of proteins were median-normalized and log_2transformed.

A one-sample Student's T-Test was performed to identify differentially expressed proteins in tissue and serum samples from endometriosis patients vs. controls. Significance was set at $p \leq 0.05$. Only proteins with a one-sample Student't T-Test p-value<0.05, a mean iTRAQ log2ratio higher than ±0.3 and identified with at least two unique peptides in adherence to the Paris Publication Guidelines for the analysis and documentation of peptide and protein identifications (http://www.mcponline.org/site/misc/ParisReport_Final.xhtml), were considered differentially expressed and subjected to bioinformatics analysis. All mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE partner repository with the dataset identifier PXD009090 (eutopic endometrium proteomic analysis) and PXD011091 (serum proteomic analysis).

Bioinformatics analysis

DAVID (https://david.ncifcrf.gov/), STRING (https://string-db.org/), BiNGO in Cytoscape and MetaCore (Clarivate Analytics, Philadelphia, PA, USA) were applied to differentially expressed proteins in order to identify over-represented gene ontology terms, pathway maps and direct protein interaction networks in endometriosis vs. control. P-values ≤ 0.05 were considered significant.

Results

Twenty-four patients were recruited between September 2013 and September 2015. Of these, eutopic endometrium from 16 subjects was used for the tissue proteomic analysis (n=8 patients with endometriosis; n=8 controls) and serum from eight subjects for the serum proteomics analysis (n=4 patients with endometriosis; n=4 controls). The clinical characteristics of the participants are presented in **Table 1**. All patients were in the proliferative phase and had a regular menstrual cycle. There was no significant difference in age, body mass index, and baseline FSH between the two groups.

Tissue and serum proteomic analysis

Tissue proteomic analysis resulted in the profiling of 10,929 proteins whereas serum proteomic analysis quantitatively identified 2,010 proteins (peptide FDR $p<0.05$). Of these, 1,214 (**Supplementary Table 1**) and 404 (**Supplementary Table 2**) were identified as differentially expressed at the tissue and serum level respectively and were further subjected to bioinformatics analysis. Forty-four DEPs were common between the two matrices, 21 of which with the same trend of differential expression (i.e. up-regulated or down-regulated in endometriosis vs. control at both tissue and serum level).

DAVID gene ontology analysis of the tissue and serum DEPs showed a significant enrichment for gene ontology terms related to Immune response | Inflammation, Cell adhesion | Migration, Blood coagulation and other terms (e.g. receptor-mediated endocytosis, high-density lipoprotein particle remodelling and G2/M transition of mitotic cycle) in both matrices

(**Figure 2A**). Forty-four DEPs were observed at both tissue and serum level and these are presented in heatmap format in **Figure 2B**. The 21 proteins with the same trend of modulation at both tissue and serum level are highlighted in grey. Ingenuity Pathway Analysis showed that carbohydrate | lipid metabolism and organ development protein networks were enriched in the 21 DEPs analysed in tissue and serum of patients with endometriosis vs. control (**Figure 3**).

Discussion

The present study reports the integrated quantitative proteomic profiling of eutopic endometrial tissue and non-depleted serum from women diagnosed with endometriosis compared to healthy controls. Bioinformatics analysis of differentially expressed proteins (DEPs) showed a significant enrichment for processes related to immune response/inflammation, cell adhesion/migration, blood coagulation in both matrices, in keeping with the known inflammatory and adhesive nature of endometriosis.

Abnormalities in immune responses have been suggested to play an important role in the perpetuation of endometriosis (36, 37). Endometrial cells in the peritoneal cavity can escape clearance from immune cells through a mechanism coined as “immunoescaping” (38). Dysregulation of immune response can thus allow the proliferation, implantation and angiogenesis of ectopic endometrial tissue (39). Furthermore, previous studies of peritoneal fluid from patients with endometriosis have shown disease-related abnormalities in the immune response (40, 41).

Studies have shown that eutopic endometrial stromal cells from females with endometriosis exhibit an altered cell-adhesion molecular profile compared to stromal cells from healthy controls (42). Extracellular matrix has been shown to control cell proliferation, differentiation and apoptosis (43).

Two proteins were found to be up-regulated in both the eutopic endometrium and serum proteomic analysis of patients with endometriosis vs. control, Na(+)/H(+) exchange regulatory cofactor NHERF-1 (NHERF-1) (gene name SLC9A3R1) and thymosin beta-4 (Tb4) (gene

name TMSB4X). Increased expression of a particular protein is more easily and reliably detected compared to lower expression levels, thus these two proteins may represent the most promising serological markers of endometriosis for further larger scale investigative studies.

NHERF-1 is a scaffold protein expressed primarily in the plasma membrane of polarized epithelial cells and mediates signals connecting the membrane to the cytoskeleton. The role of NHERF-1 in uterine physiology remains unknown, with few studies reporting its involvement with pathological conditions such as endometrial cancer and polycystic ovaries syndrome (PCOS). NHERF-1 contributes to the organization of microvilli in polarized epitheliums, but also regulates the activity of growth factor receptors, ion channels and the endocytic machinery (44-46). A study showed that NHERF-1 expression is transcriptionally regulated by oestrogens in human endometrium, and that it is expressed at higher levels during the proliferative phase of the menstrual cycle (47). The role of NHERF-1 in endometriosis warrants further investigation.

Tb4, a member of the beta-thymosins family, is an N-terminally acetylated peptide composed of 43 amino acid residues (48). Tb4 interacts with monomeric actin (48) and modulates actin polymerization (49). As a secreted factor, Tb4 has been found to modulate the immune response and participate in hormonal activities (48, 50). Tb4 is also involved in inflammatory response, angiogenesis, blood coagulation, wound healing and apoptosis (51-54). Using a mouse model, Kawahara *et al.* (55) showed that Tb4 over-expression could participate in musculature disintegration and the development of adenomyosis. The role of Tb4 in endometriosis should be assessed in future studies.

The main limitation of the study is its small size. Power calculation of sample size (n=16 for tissue analysis and n=8 for serum analysis) was based on ensuring a statistical power of over 0.7, taking into consideration a 30% measurement error and a $\log_2$ratio fold change > 0.3 between biological replicates, as reported in a similar simulation study (56). Validating the proteins at the tissue and serum level in a larger cohort using mass spectrometry or an alternative analytical method to mass spectrometry (e.g. ELISA or western blot) to confirm

their clinical utility was beyond the scope of the present study and constitutes a future perspective.

In conclusion, the integrated eutopic endometrium and serum global proteomic profiling identified candidate serological targets that can be further validated for their clinical utility in the non-invasive diagnosis of endometriosis.

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Disclosure of interests

The authors declare no conflict of interest

Contribution to Authorship

AM performed experiments, analysed/interpreted data and wrote manuscript; MH collected samples, performed experiments, analysed/interpreted data; MF, DJGB and JT performed experiments and analysed data; SDG and YC designed study, supervised the execution of experiments, interpreted the experimental results and wrote manuscript.

Details of ethics approval

This study has institutional and regional review board approval by the University Hospital Southampton (RHMO&G160) and Hampshire B ethical committees (MREC08/ HO502/162) (Approval date: 24 October 2008).

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Table and Figure Legends

Table 1. Clinical characteristics of study participants

Figure 1. Study design

Figure 2. A. DAVID gene ontology analysis of the DEPs showed a significant enrichment for gene ontology terms related to Immune response | Inflammation, Cell adhesion | Migration, Blood coagulation in both tissue and serum from patients with endometriosis vs. control. **B**. Heatmap of fourty-four DEPs observed at both tissue and serum level. Proteins with the same trend of modulation at both matrices are highlighted in grey.

Figure 3. Ingenuity Pathway Analysis showed that carbohydrate | lipid metabolism and organ development protein networks were enriched in the 21 DEPs analysed in tissue and serum of patients with endometriosis vs. control