

**DNA methylation alterations - potential cause of endometriosis pathogenesis or a reflection of tissue heterogeneity?**

Merli Saare<sup>1,2</sup>; Kertu Liis Krigul<sup>3</sup>; Triin Laisk-Podar<sup>1,2</sup>, Sakthi Vignesh-Srinivasan<sup>4</sup>; Nilufer Rahmioglu<sup>5,6</sup>; Parameswaran Grace Lalit Kumar<sup>4</sup>, Krina Zondervan<sup>5,6</sup>; Andres Salumets<sup>1,2,7,8</sup>; Maire Peters<sup>1,2</sup>

<sup>1</sup>Competence Centre on Health Technologies; <sup>2</sup>Institute of Clinical Medicine, Department of Obstetrics and Gynecology, University of Tartu <sup>3</sup>Institute of Molecular and Cell Biology, University of Tartu; <sup>4</sup>Division of Obstetrics and Gynecology, Department of Women's and Children's Health, Karolinska Institutet; <sup>5</sup>Wellcome Centre for Human Genetics, University of Oxford; <sup>6</sup>Endometriosis CaRe Centre, Nuffield Department of Obstetrics & Gynaecology, John Radcliffe Hospital, University of Oxford; <sup>7</sup>Department of Obstetrics and Gynecology, University of Helsinki and Helsinki University Hospital; <sup>8</sup>Institute of Bio- and Translational Medicine, University of Tartu.

Corresponding author: [merli.saare@ut.ee](mailto:merli.saare@ut.ee)

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Summary sentence: Cellular heterogeneity of endometriotic lesions and endometrial biopsies has significant impact on the results of DNA methylation studies in endometriosis

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## 25    **Abstract**

26    Alterations in the DNA methylation pattern of endometriotic lesions and endometrium of  
27    endometriosis patients have been proposed as one potential factor accompanying the endometriosis  
28    development. Although many differentially methylated genes have been associated with the  
29    pathogenesis of this disease, the overlap between the results of different studies has remained  
30    small. Among other potential confounders, the impact of tissue heterogeneity on the outcome of  
31    DNA methylation studies should be considered, as tissues are mixtures of different cell-types with  
32    their own specific DNA methylation signatures. This review focuses on the results of DNA  
33    methylation studies in endometriosis from the cellular heterogeneity perspective. We consider both  
34    the studies using highly heterogeneous whole-lesion biopsies and endometrial tissue, as well as  
35    pure cell fractions isolated from lesions and endometrium to understand the potential impact of the  
36    cellular composition to the results of endometriosis DNA methylation studies. Also, future  
37    perspectives on how to diminish the impact of tissue heterogeneity in similar studies are provided.

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## Introduction

DNA methylation is a common epigenetic process, occurring mainly in CpG dinucleotide-rich areas, referred as CpG islands, where the cytosine nucleotide is converted by DNA methyltransferases into 5'-methylcytosine (5mC). Large majority of annotated gene promoters are associated with CpG islands and methylation of these islands may result in down-regulation or silencing of the gene expression [1]. Thus, the DNA methylation has crucial role in regulation of many fundamental cellular processes, including genome regulation, development and maintenances of tissue-specific gene expression pattern. Alterations disturbing DNA methylation/gene expression may initiate pathologic processes and contribute to the development of many diseases [2]. In recent years, alterations in the DNA methylation process have also been considered as one possible mechanism behind endometriosis development.

In addition, a functionally different type of DNA modification, namely hydroxymethylation, has recently been described [3,4]. Ten-eleven translocation (TET) proteins are responsible for creating 5-hydroxymethylcytosine (5hmC) [4] that is most abundant within the central nervous system, but has also been detected in other tissues, including endometrium [5–8] and can contribute to the regulation of gene expression in endometriosis.

However, the number of studies evaluating the DNA methylation profiles in endometriosis has remained relatively moderate and only first attempts have been done to elucidate the possible role of hydroxymethylation in the disease development. To date, around 30 studies using both candidate gene-based and epigenome-wide association studies (EWAS) approaches have been conducted to uncover the DNA methylation changes in endometriotic lesions, in endometrium and also in *in vitro* cultured endometrial stromal cells from lesions and endometrium (Table 1-3).

Despite the fact that these studies have brought out a large number of differentially methylated genes (e.g. *NR5A1*, *HOXA10*, *PGR*, *GATA2*) that could be associated with disease initiation, progression and pathogenesis, the overlap between the results of different studies has remained small and alterations in DNA methylation patterns reported in one study are seldom confirmed by others. As the understanding about the nature of differential DNA methylation in different tissues and cell types is constantly evolving and there is clear evidence about the normal variability in DNA-methylation signature in different tissues and cells [9–11], it can be assumed that one potential reason for varying results of endometriosis studies may be hidden in the tissue/cell type heterogeneity (Figure 1). In endometriosis studies, different strategies have been applied and tissues with different level of cellular heterogeneity have been used. Some studies have compared whole-lesion biopsies with endometrial tissue; however, the cellular composition of endometriotic lesions is highly heterogeneous, as endometrial stromal and epithelial cells are mixed with cells from surrounding tissue (peritoneal tissue, ovarian components etc.) and tissue-infiltrated blood cells in variable proportions. Therefore, the amount of disease-specific cells in lesions may have a crucial impact on the outcome of DNA methylation analysis and may lead to inconsistent or wrongly interpreted results. Some studies have analysed moderately heterogeneous endometrial tissue, with no cellular contribution from other tissues, from patients and controls, and some have used pure endometrial stromal cell populations isolated from lesions and endometrium, as the study material with the lowest cellular heterogeneity. In this review, the results of DNA methylation and hydroxymethylation studies in endometriosis are discussed in the perspective of cellular heterogeneity, considering studies using high-, moderate- and low heterogeneity samples.

## High tissue/cellular heterogeneity - DNA methylation studies in endometriotic lesion whole-tissue biopsies

Highly heterogeneous endometriotic lesions, which are removed during laparoscopic surgery, have been an attractive study object for both candidate gene-based and genome-wide DNA methylation studies [12–26]. Up to date, DNA methylation profiles of more than 10 candidate genes and transposable element involved in different pathways, such as hormonal signalling (*PGR*, *ESR1*, *ESR2*, *COX-2*, *COMT*), ovarian cancer progression (*LINE-1*), carcinogenesis (*PAX2*), tumour repressor and apoptosis-related genes (*CDH1*, *RASSF*), tissue remodelling (*MMP2*, *MMP3*, *MMP7*, *TIMP3* and *TIMP4*) and genes needed for endometrial growth, differentiation, and implantation (*HOXA10*) have been investigated and associated with disease pathogenesis in whole endometriotic lesion biopsies (Table 1).

However, there are only two EWAS investigating DNA methylation in whole lesion tissues [24,25]. Borghese et al. (2010) evaluated the DNA methylation status of more than 25,000 promoters using MeDIP-chip technology to ascertain the methylation profile of different type of endometriotic lesions (superficial endometriosis, ovarian cysts, and deeply infiltrating endometriosis). The study compared DNA methylation of pooled DNA samples from 15 eutopic endometria to pooled DNA samples from different lesion-types and detected 229, 161, and 108 differentially methylated regions in superficial endometriosis, ovarian cysts, and deeply infiltrating endometriosis, respectively. Some of the genes (*FLJ38379*, *DEFB125*, *GOLGB1*, *PERP*, *NOM1*, *CNTLN*, *RASSF4*, *C10orf25*, *ZNF22*, *HRAS*, *LRRC56*, *F7*, *DKFZp451A211*, *ADPRHL1*, *TPSD1*, *PYDC1*, *TEX14*, *RAD51C*, *RNF126*, *FSTL3* and *FTHL19*) were differentially methylated in all lesion subtypes. However, it should be pointed out that this work did not confirm the differential

methylation of previously reported candidate genes. Authors suggested that this was because of the use of highly specific microarray with limited capacity to detect low CpG- containing regions. However, the most intriguing finding of this study was that in endometriotic lesions hypomethylated regions were distributed randomly across the chromosomes, whereas hypermethylated regions tended to locate at the ends of the chromosomes. The authors proposed that noticed asymmetric methylation pattern enhances chromosome stability and presume endometriotic cells from malignant transformation [24]. In the most recent study, Rahmioglu et al. [25] analysed the DNA methylation profiles of 14 endometriotic lesions (both endometriomas and peritoneal lesions) and 16 endometrial samples from endometriosis patients and found 27,493 significantly differentially methylated sites corresponding to 8,133 genes. Among these genes was a significant enrichment for *WNT* signalling, angiogenesis, cadherin signalling and gonadotropin-releasing-hormone-receptor pathways that have been previously associated with endometriosis pathogenesis. The authors brought out that endometrium, endometriomas and peritoneal lesions have their distinct DNA methylation signatures and suggested that whole-tissue profiling will detect robust DNA methylation between the individuals, but for low-variability DNA methylation sites the cellular heterogeneity and technical variability hinder the detection of biologically meaningful alterations. Furthermore, significant impact of menstrual cycle phases on endometrial and peritoneal lesions DNA methylation signature was noticed, but in endometriomas, this effect was less pronounced [25].

The studies concerning hydroxymethylation level in endometriosis whole tissues have given contradictory results, most probably because of different methodologies used to detect the amount of 5hmC. Using an ELISA-based colorimetric quantification, it was found that DNA of whole endometriotic tissues contains very high amounts of 5hmC compared to non-endometriosis

control eutopic endometrium, a phenomenon corroborated by the inverse expression of TET genes in these tissues [5]. In the following immunofluorescence study, Yotova et al. showed that on the whole tissue level there was a disease-dependent loss of 5hmC in the endometriotic tissue epithelial cells but not in the stromal cell compartment [6].

The high heterogeneity of endometriotic lesions' cellular composition and lack of knowledge about the normal DNA methylation profiles of surrounding tissues and cells makes the discovery of disease specific changes extremely difficult and even if thousands of differentially methylated genes have been reported, it is almost impossible to distinguish whether these findings reflect the complex mixed cellular composition of lesions or these alterations are truly endometriosis-related changes.

#### **Moderate tissue/cellular heterogeneity - DNA methylation studies of endometrium and potential impact of menstrual cycle on endometrial methylome**

The theory of endometrial origin of endometriosis, which postulates that endometrial cells are refluxed via retrograde menstruation and implant into the abdominal cavity and form ectopic lesions, is widely accepted [27]. However, for successful establishment of lesions, endometrial cells of endometriosis patients should have altered characteristics triggering the endometrial cell adhesion and growth in the ectopic locations. This assumption is supported by multiple lines of evidence showing that endometria of endometriosis patients have aberrant gene expression profiles compared to healthy women [28–30]. As DNA methylation is a potential cause of gene expression alterations, it has encouraged researchers to seek for the methylation changes in patient's endometria. Although the endometrium is a mixture of different cell types, where endometrial apical and glandular epithelial and stromal cells are mixed with tissue-infiltrated blood cells, the

cellular heterogeneity between endometrial samples from patients and controls is definitely less pronounced than between endometrium and lesion biopsies, facilitating the identification of true disease-related DNA methylation changes. Still, the search for disease-related endometrium-specific DNA methylation alterations is a challenging task because of extensive molecular, morphological and physiological changes occurring during the menstrual cycle. Furthermore, the impact of menstrual cycle phases on the DNA methylation profile in healthy women's endometrial tissue has recently been demonstrated [31,32].

The candidate gene-based and DNA microarray studies comparing endometria from women with and without endometriosis have brought out number of disease related genes (Table 2) [12,15,16,18,19,33–42]. The candidate gene-based studies have reported dysregulation of genes involved in many important biological functions, such as hormonal regulation (*COX-2*), development of female genital tract (*PAX2*), endometrial growth and receptivity (*HOXA10*, *HOXA11*) and tumor suppression (*RUNX3*, *RASSF1A*, *CDH1*) [12,33,34,36,38,39,43,44].

In addition to candidate gene studies, four EWAS comparing endometria from healthy women and endometriosis patients have been published [25,40–42]. The study by Naqvi et al. [40] investigated 27,578 CpGs in 7 women with endometriosis and 6 controls and found 120 statistically significant differentially methylated genes (59 hypermethylated and 61 hypomethylated). They also confirmed correlations between methylation status and gene expression level among a set of ten selected genes and proposed that these genes may contribute to the abnormal regulation of endometrial cell proliferation in women with endometriosis. However, our recent study suggested that methylation profiles between endometria of patients (n=31) and controls (n=24) are highly similar [41]. We found only 28 differentially methylated regions, from which 16 were associated with known genes - *PI3*, *SLC43A3I*, *MGAT5B*, *MUC4*,



*HIVEP3*, *FGG*, *CLCF1*, *CANT1*, *LTK*, *AHRR*, *AKR1B1*, *APEH*, *CST11*, *ELOVL4*, *HBE1* and  
*NEGR1*. Furthermore, the overall magnitude of methylation differences was rather small, and as  
the biological significance of small-scale changes in DNA methylation levels is currently  
unknown, it is unclear whether and how these changes are associated with disease pathogenesis.  
In addition, methylation status of some of these genes, such as *PI3*, *SLC43A3I*, *MGAT5B*, *MUC4*,  
*HIVEP3*, *FGG*, *CANT1* and *LTK* was influenced by menstrual cycle phase, indicating that it is  
crucially important to take into account the normal epigenetic changes across the menstrual cycle  
when looking for disease specific methylation differences in endometrial tissue [41]. The  
menstrual cycle dependent endometrial DNA methylation was confirmed also by Houshdaran et  
al. [42] who found that the DNA methylome differences between the endometriosis patients (n=17)  
and controls (n=16) were most contrasting in the mid-secretory phase, at the time of the  
progesterone peak (137 CpG sites, corresponding to 125 loci), followed by proliferative (58 CpG  
sites, corresponding to 58 loci) and early-secretory phase (39 CpG sites, corresponding to 36 loci).  
Moreover, if all patient samples were compared to all control samples regardless the menstrual  
cycle phase, only three differentially methylated loci (*RPF2*, *PER1* and *FAM181A*) remained.  
Locus in gene *RPF2* was more methylated in controls while loci in *PER1* and *FAM181A* were  
more methylated in endometriosis patients [42]. Menstrual cycle phase as an important covariate  
in DNA methylation and RNA expression analysis was also reported by Rahmioglu et al. [25],  
who found no significant changes between the endometrial DNA methylation profiles of women  
with and without endometriosis but reported significant variability of DNA methylation between  
the different menstrual cycle phases.

While we compared all available data from the findings of genome-wide studies  
demonstrating any changes in endometrial DNA methylation in endometriosis [40–42], we found

no common genes, indicating that DNA methylation changes in endometrium are probably not the main trigger leading to endometriosis development. On the other hand, it is possible that the confounding factors, like menstrual cycle, study design, differences in data analysis and interpretation mask small but relevant changes. Furthermore, power calculations revealed that most of the so far performed studies have been unpowered to detect reliable methylation differences between the groups [25]. For example, to detect 2% ( $\Delta\beta=0.02$ ) DNA methylation difference between cases and controls, at least 500 patient's samples are needed [25].

#### **Low tissue/cellular heterogeneity - DNA methylation studies of endometrial primary stromal cells**

Investigation of primary cell cultures enables to minimise the confounding effects of the accompanying cells in the tissues and to compare the same type of cells irrespective of the original location. The use of primary cells from lesions and endometrium in DNA methylation studies has its own pros and cons as discussed below, but still offers a good solution to study the molecular mechanisms of endometriosis with minimal impact of cellular heterogeneity.

To date, there have been nine studies (six candidate-gene and three EWAS) determining the DNA methylation profiles of primary stromal cells from endometrium and lesions [45–51,6,52] (Table 3). Closer look at the candidate-gene studies reveals that all studies have been focused on the genes participating in the hormonal regulation of the endometrial cells, such as *NR5A1*, *ESR2*, *CYP19* and *DUSP2*. The most commonly studied gene *NR5A1* [45–47] encodes transcription factor steroidogenic factor 1 (SF-1) that has a role in activating *STAR* and aromatase production

and therefore could potentially contribute to the higher level of oestrogens in endometriotic stromal cells [53]. Interestingly, methylation status of distinct regions of *NR5A1* influences its expression in different ways. Hypermethylation of the proximal promoter of *NR5A1* in endometrial stromal cells is accompanied by the lack of *NR5A1* expression, and on the opposite, the hypomethylation of the same region in endometriotic cells leads to the high expression of *NR5A1* mRNA, confirming the general understanding that DNA methylation of promoter region is inversely correlated with transcription [47]. However, the following studies revealed that hypermethylation of CpG islands in introns and exon 2 leads to the high expression of *NR5A1* mRNA in endometriotic cells [46,54] indicating that methylation of CpG islands outside the promoter region may also play an important role in regulating *NR5A1* expression.

Beside candidate-gene studies, three EWAS methylation studies have been conducted concentrating on stromal cells from ovarian cysts and eutopic endometria from endometriosis patients and healthy women. As a result, a large number of potentially disease-related differentially methylated genes were detected (9,021, 770 and 43, respectively) [50,6,52]. When we compared the lists of genes that were reported in all three studies, only a small subset of overlapping genes (*S100A4*, *RBM24*, *GATA2*, *DAPK1*, *NR5A1*, *C11orf9*, *CCL26* and *GATA4*) was found. Some of the genes from this list are particularly interesting. For example, the GATA family members have previously been associated with induction of “ovarian-like” differentiation of ectopic endometrial cells [55]. It has been suggested that similarly to the ovarian cells, the expression of *GATA4* and *GATA6* transcription factors is induced via the positive feedback loop by the follicle stimulating hormone receptor (FSHR) and luteinizing hormone receptor (LHR) genes also in ectopic endometrial cells. Thus, the parallel increase in GATA4/6 transcription factors and FSHR and LHR levels leads to enhanced production of the steroidogenic cascade that supports oestrogen-

dependent disease progression [55]. Furthermore, the hypermethylation and downregulation of *GATA2* regulates strongly the genes essential for decidualization, and hypomethylation and activation of *GATA6* promotes an endometriotic phenotype through progesterone resistance and altered oestrogen response [50]. Also, all microarray-based studies have reported hypermethylation of fibroblast-specific protein-1 (*S100A4*). The expression of this gene is shown to be induced by the TGF- $\beta$  pathway, and silencing of *S100A4* expression can inhibit the process of TGF- $\beta$ -induced epithelial-mesenchymal transition (EMT) [56]. As during the EMT process epithelial cells lose their specific features and acquire more invasive mesenchymal characteristics, the EMT is believed to be a crucial event leading to the development of endometriosis [57].

It is worth mentioning that the differential methylation and expression of *NR5A1* seen in candidate-gene studies was proven in EWAS studies. Moreover, it was confirmed that not only the promoter-region hypomethylation regulates the expression of this gene [50,52] but that the intronic region hypermethylation in endometriotic stromal cells allows for higher level of *NR5A1* expression [50].

The first study assessing the role of 5hmC in endometrial and endometriotic stromal cells [6] demonstrated that detected altered methylation of *BDNF* gene in the EWAS was partly caused by hydroxymethylation [6]. Still, to understand the exact role of 5hmC in endometriosis development and endometrial biology, future studies elucidating the changes of 5hmC in different cell types obtained from the endometrium or endometriotic lesions and along the menstrual cycle, are urgently needed.

The studies on isolated stromal cells have found the greatest number of overlapping differentially methylated and expressed genes, demonstrating the benefit of investigating homogenous cell populations. However, it should be pointed out that the use of primary cells from

lesions and endometrium in DNA methylation studies has its own strengths and weaknesses. On the one side, primary cells are phenotypically and epigenetically similar to the same cell-type in the tissue of origin, but on the other side, the natural environment of the cells is destroyed in cellular separation with cells adapting quickly to *in vitro* conditions. The *in vitro* environment with supporting hormonal milieu is especially important in case of hormone-dependent cells such as endometrial cells. Furthermore, primary cell culturing and repeated passaging changes the cell subpopulation dynamics and leads to alterations in their whole transcriptome [58,59] and in DNA methylation [60]. Thus, to keep the molecular signatures as similar as possible to the original tissue, the studies of the primary cells should be limited only to the early passages [58,59]. Furthermore, although it is generally accepted that primary cell culture is a homogenous mixture of identical cells, most of the primary cultures include also a small fraction of other cell-types from the same tissue, ranging usually around 1-5% [61–64]. The issue of cellular contamination becomes particularly important for establishing slowly proliferating epithelial cell culture as even a small contamination with highly proliferative stromal cells may largely change the cellular composition of the culture. Although large efforts have been made to culture primary endometrial epithelial cells from endometrium, these cells can only be passaged once [65,66] with cell senescence becoming evident already within 2 weeks in culture [67]. Therefore, as culturing of endometrial epithelial cells has still remained a challenge, no specific studies on DNA methylation alterations in endometrial epithelial cells from endometriotic lesions exist.

Moreover, in case of isolation and culturing of primary endometrial stromal cells from endometriomas, it is impossible to identify the possible ovarian stromal contamination due to the lack of specific markers [68], creating additional bias in endometrioma studies. Last but not least, DNA methylation studies on isolated stromal cells of lesions have thus far been concentrated only

on cells isolated from endometriomas. Whether there are also DNA methylation alterations in cells isolated from peritoneal lesions, is currently unknown but definitely worth to study. Therefore, in order to eliminate the bias coming from cell culturing and potential contamination of the primary cell culture, we suggest using uncultured cells from lesions and endometrium to reveal the true molecular differences occurring inside the lesions, as discussed in the next paragraph.

### **Possible approaches to diminish cellular heterogeneity in endometriosis studies**

To diminish cellular heterogeneity in endometriosis studies and analyse DNA methylation of endometrium-specific cells with minimal impact of cells from surrounding tissues, several solutions could be proposed. First, specific cell populations can be isolated from histological tissue sections, such as formalin-fixed and paraffin-embedded (FFPE) tissues, RNAlater stored or snap-frozen tissues, by laser capture microdissection (LCM). Although this methodology has been suggested to be time-consuming, labour-intensive and providing only limited amount of DNA for methylation analysis, it still represents the most effective technology to isolate a morphologically homogeneous population of specific cells. However, the attractiveness of this methodology has remained small and to date, there are no DNA methylation studies in endometriosis that had used the potential advantage of LCM. It is very likely that small interest of using LCM for isolation of specific cell populations is related to the poor quality of DNA obtained, especially when the DNA is isolated from archived FFPE tissues. For example, LCM DNA from snap-frozen sections allows to amplify DNA regions of >300 bp, while from FFPE tissues only 150 to 200 bp fragments are available [69]. However, cancer studies have clearly proven the usefulness of LCM in DNA methylation analysis [70].

Second, the uncultured single cells or cell populations from lesion biopsies and endometrium can be isolated using fluorescently labelled antibodies against cell surface markers in combination with fluorescence activated cell sorting (FACS). FACS methodology has previously been used to isolate specific cell types from endometrium of healthy women [71] and from endometrium of women suffering from endometriosis [72,73] for gene expression studies. We have shown the usefulness of this methodology for isolation of CD10-positive stromal cells from endometrioma biopsies for transcriptome study [72] and the amount of cells obtained by FACS should also be suitable for DNA methylation analysis. Currently, the main limitation of this methodology is the absence of specific antibodies to discriminate endometrial epithelial cells from lesion biopsy. While the anti-CD10 antibody discriminates CD10-negative ovarian stromal cells from CD10-positive endometrial stromal cells [74], the previously used endometrial epithelial cell-specific anti-CD9 antibody [71] does not distinguish them from similar CD9-positive granulosa and epithelial cells from endometrioma samples [75]. Furthermore, it should be kept in mind that beside endometrial epithelial and stromal cells, lesions also contain other cell types and DNA methylation changes in these cells may have significant impact on disease pathogenesis and deserve further investigation.

Thirdly, also computational approaches for tackling cellular heterogeneity can be used. As already stated, each tissue or cell type has a specific methylation signature and fluctuations in biopsy cellular composition can dramatically confound analyses, either by creating false positive associations (e.g. the detected differences actually simply tag the change in cellular composition) or masking actual associations. To overcome this obvious limitation in EWAS, several computational approaches have been developed, which allow to adjust for cellular heterogeneity, an approach known as cell-type deconvolution (reviewed by [76]). In broad terms, these

deconvolution methods can be either reference-based or reference free. The former approach uses reference methylation profiles for cell types present in the tissue and works under the assumption that the methylation profile of a whole-tissue biopsy is a sum of reference profiles of each cell type present in this biopsy. As a result, the algorithm calculates the fractions for each cell type that match the whole-tissue biopsy profile best. These fractions can then be used as covariates in differential methylation analysis to adjust for differences in whole-tissue biopsy cellular composition. A major drawback for using this approach in the context of endometriosis studies is the lack of suitable reference profiles for cell types present in the endometrial tissue or endometriotic lesions.

As an alternative, reference-free deconvolution methods, such as EWASher [77], RefFreeEWAS [78], and ReFACTor [79] or surrogate variable analysis (SVA) that allow to adjust for fluctuations in cellular composition can be used. Selection of the best method depends on study design and research question, but SVA was shown to be the most stable and robust method across different scenarios [80]. However, currently no study has compared the performance of these different methods in the context of endometriosis. Therefore, since it is clear that tissue heterogeneity is a very important confounder in endometriosis research, there is a critical need to describe accurately the cellular (sub)populations present in endometrium and endometriotic lesions to generate high-quality reference methylomes for these cell types, and to compare the performance of different deconvolution algorithms for endometrium and endometriotic lesions. These bits would provide the necessary basis to tackle the cellular heterogeneity on a computational level and provide a viable alternative to analyzing separate cell populations, which is labor-intensive and costly.



## **Conclusions**

The first steps to unravel the role of altered DNA methylation and hydroxymethylation in endometriosis development have been done but there is a long way to go before we can ascertain whether the knowledge gained from these studies could be benefitted in improving the endometriosis diagnostics or therapy. To overcome the issues related to tissue biopsy heterogeneity, the methylation differences should be demonstrated in pure populations of cells and a direct link between the methylation and gene expression alterations in the same cells should be established.

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## References

- [1] Deaton AM, Bird A. CpG islands and the regulation of transcription. *Genes Dev* 2011; 25:1010–22.
- [2] Bergman Y, Cedar H. DNA methylation dynamics in health and disease. *Nat Struct Mol Biol* 2013; 20:274–281.
- [3] Kriaucionis S, Heintz N. The nuclear DNA base 5-hydroxymethylcytosine is present in Purkinje neurons and the brain. *Science* 2009; 324:929–30.
- [4] Tahiliani M, Koh KP, Shen Y, Pastor WA, Bandukwala H, Brudno Y, Agarwal S, Iyer LM, Liu DR, Aravind L, Rao A. Conversion of 5-methylcytosine to 5-hydroxymethylcytosine in mammalian DNA by MLL partner TET1. *Science* 2009; 324:930–5.
- [5] Roca FJ, Loomans HA, Wittman AT, Creighton CJ, Hawkins SM. Ten-Eleven Translocation Genes are Downregulated in Endometriosis. *Curr Mol Med* 2016; 16:288–98.
- [6] Yotova I, Hsu E, Do C, Gaba A, Sczabolcs M, Dekan S, Kenner L, Wenzl R, Tycko B. Epigenetic Alterations Affecting Transcription Factors and Signaling Pathways in Stromal Cells of Endometriosis. *PLoS One* 2017; 12:e0170859.
- [7] Nestor CE, Ottaviano R, Reddington J, Sproul D, Reinhardt D, Dunican D, Katz E, Dixon JM, Harrison DJ, Meehan RR. Tissue type is a major modifier of the 5-hydroxymethylcytosine content of human genes. *Genome Res* 2012; 22:467–77.
- [8] Globisch D, Münzel M, Müller M, Michalakakis S, Wagner M, Koch S, Brückl T, Biel M, Carell T. Tissue distribution of 5-hydroxymethylcytosine and search for active demethylation intermediates. *PLoS One* 2010; 5:e15367.

- 406 [9] Varley KE, Gertz J, Bowling KM, Parker SL, Reddy TE, Pauli-Behn F, Cross MK,  
 407 Williams BA, Stamatoyannopoulos JA, Crawford GE, Absher DM, Wold BJ, et al.  
 408 Dynamic DNA methylation across diverse human cell lines and tissues. *Genome Res*  
 409 2013; 23:555–67.
- 410 [10] Roadmap Epigenomics Consortium A, Kundaje A, Meuleman W, Ernst J, Bilenky M, Yen  
 411 A, Heravi-Moussavi A, Kheradpour P, Zhang Z, Wang J, Ziller MJ, Amin V, et al.  
 412 Integrative analysis of 111 reference human epigenomes. *Nature* 2015; 518:317–30.
- 413 [11] Lokk K, Modhukur V, Rajashekar B, Martens K, Magi R, Kolde R, Koltsina M, Nilsson  
 414 TK, Vilo J, Salumets A, Tonisson N. DNA methylome profiling of human tissues  
 415 identifies global and tissue-specific methylation patterns. *Genome Biol* 2014; 15:r54.
- 416 [12] Zidan HE, Rezk NA, Alnemr AAA, Abd El Ghany AM. COX-2 gene promoter DNA  
 417 methylation status in eutopic and ectopic endometrium of Egyptian women with  
 418 endometriosis. *J Reprod Immunol* 2015; 112:63–7.
- 419 [13] Senthong A, Kitkumthorn N, Rattanatanyong P, Khemapech N, Triratanachart S,  
 420 Mutirangura A. Differences in LINE-1 methylation between endometriotic ovarian cyst  
 421 and endometriosis-associated ovarian cancer. *Int J Gynecol Cancer* 2014; 24:36–42.
- 422 [14] Ren F, Wang D-B, Li T, Chen Y-H, Li Y. Identification of differentially methylated genes  
 423 in the malignant transformation of ovarian endometriosis. *J Ovarian Res* 2014; 7:73.
- 424 [15] Wu YU, Zhang M, Zhang X, Xu Z, Jin W. Methylation status and protein expression of  
 425 RASSF1A in endometriosis. *Oncol Lett* 2016; 11:4107–4112.
- 426 [16] de Graaff AA, Delvoux B, Van de Vijver KK, Kyama CM, D’Hooghe TM, Dunselman  
 427 GAJ, Romano A. Paired-box gene 2 is down-regulated in endometriosis and correlates  
 428 with low epidermal growth factor receptor expression. *Hum Reprod* 2012; 27:1676–84.

- 429 [17] Wu Y, Strawn E, Basir Z, Wang Y, Halverson G, Jailwala P, Guo SW. Genomic  
430 alterations in ectopic and eutopic endometria of women with endometriosis. *Gynecol*  
431 *Obstet Invest* 2006; 62:148–159.
- 432 [18] Li Y, An D, Guan Y-X, Kang S. Aberrant Methylation of the E-Cadherin Gene Promoter  
433 Region in Endometrium and Ovarian Endometriotic Cysts of Patients with Ovarian  
434 Endometriosis. *Gynecol Obstet Invest* 2017; 82:78–85.
- 435 [19] Andersson KL, Bussani C, Fambrini M, Polverino V, Taddei GL, Gemzell-Danielsson K,  
436 Scarselli G. DNA methylation of HOXA10 in eutopic and ectopic endometrium. *Hum*  
437 *Reprod* 2014; 29:1906–1911.
- 438 [20] Ji F, Yang X, He Y, Wang H, Aili A, Ding Y. Aberrant endometrial DNA methylome of  
439 homeobox A10 and catechol-O-methyltransferase in endometriosis. *J Assist Reprod Genet*  
440 2017; 34:409–415.
- 441 [21] Meyer JL, Zimbardi D, Podgaec S, Amorim RL, Abrão MS, Rainho CA. DNA  
442 methylation patterns of steroid receptor genes ESR1, ESR2 and PGR in deep  
443 endometriosis compromising the rectum. *Int J Mol Med* 2014; 33:897–904.
- 444 [22] Zhou H, Li J, Podratz KC, Tipton T, Marzolf S, Chen H Bin, Jiang S-W. Hypomethylation  
445 and activation of syncytin-1 gene in endometriotic tissue. *Curr Pharm Des* 2014; 20:1786–  
446 95.
- 447 [23] Roesse-Koerner B, Stappert L, Berger T, Braun NC, Veltel M, Jungverdorben J, Evert BO,  
448 Peitz M, Borghese L, Brüstle O. Reciprocal Regulation between Bifunctional miR-9/9(\*)  
449 and its Transcriptional Modulator Notch in Human Neural Stem Cell Self-Renewal and  
450 Differentiation. *Stem Cell Reports* 2016; 7:207–19.
- 451 [24] Borghese B, Barbaux S, Mondon F, Santulli P, Pierre G, Vinci G, Chapron C, Vaiman D.

- 452 Research resource: genome-wide profiling of methylated promoters in endometriosis  
 453 reveals a subtelomeric location of hypermethylation. *Mol Endocrinol* 2010; 24:1872–  
 454 1885.
- 455 [25] Rahmioglu N, Drong AW, Lockstone H, Tapmeier T, Hellner K, Saare M, Laisk-Podar T,  
 456 Dew C, Tough E, Nicholson G, Peters M, Morris AP, et al. Variability of genome-wide  
 457 DNA methylation and mRNA expression profiles in reproductive and endocrine disease  
 458 related tissues. *Epigenetics* 2017; 12:897–908.
- 459 [26] Tang L, Xiang Y, Zhou Y, Mu J, Zai M, Xing Q, Zhao X, He L, Wang L, Dong X, Li Q.  
 460 The DNA methylation status of genes encoding Matrix metalloproteinases and tissue  
 461 inhibitors of Matrix metalloproteinases in endometriosis. *Mol Reprod Dev* 2018; 85  
 462 (1):17–25.
- 463 [27] Sampson JA. Metastatic or Embolic Endometriosis, due to the Menstrual Dissemination of  
 464 Endometrial Tissue into the Venous Circulation. *Am J Pathol* 1927; 3:93–110 43.
- 465 [28] Fassbender A, Verbeeck N, Bornigen D, Kyama CM, Bokor A, Vodolazkaia A, Peeraer  
 466 K, Tomassetti C, Meuleman C, Gevaert O, Van de Plas R, Ojeda F, et al. Combined  
 467 mRNA microarray and proteomic analysis of eutopic endometrium of women with and  
 468 without endometriosis. *Hum Reprod* 2012; 27:2020–2029.
- 469 [29] Tamaresis JS, Irwin JC, Goldfien GA, Rabban JT, Burney RO, Nezhat C, DePaolo L V,  
 470 Giudice LC. Molecular Classification of Endometriosis and Disease Stage Using High-  
 471 Dimensional Genomic Data. *Endocrinology* 2014;en20141490.
- 472 [30] Aghajanova L, Giudice LC. Molecular evidence for differences in endometrium in severe  
 473 versus mild endometriosis. *Reprod Sci* 2011; 18:229–251.
- 474 [31] Houshdaran S, Zelenko Z, Irwin JC, Giudice LC. Human endometrial DNA methylome is

- 475 cycle-dependent and is associated with gene expression regulation. *Mol Endocrinol* 2014;  
 476 28:1118–1135.
- 477 [32] Kukushkina V, Modhukur V, Suhorutšenko M, Peters M, Mägi R, Rahmioglu N, Velthut-  
 478 Meikas A, Altmäe S, Esteban FJ, Vilo J, Zondervan K, Salumets A, et al. DNA  
 479 methylation changes in endometrium and correlation with gene expression during the  
 480 transition from pre-receptive to receptive phase. *Sci Rep* 2017; 7:3916.
- 481 [33] Kulp JL, Mamillapalli R, Taylor HS. Aberrant HOXA10 Methylation in Patients With  
 482 Common Gynecologic Disorders: Implications for Reproductive Outcomes. *Reprod Sci*  
 483 2016; 23:455–63.
- 484 [34] Wu Y, Halverson G, Basir Z, Strawn E, Yan P, Guo SW. Aberrant methylation at  
 485 HOXA10 may be responsible for its aberrant expression in the endometrium of patients  
 486 with endometriosis. *Am J Obstet Gynecol* 2005; 193:371–380.
- 487 [35] Szczepańska M, Wirstlein P, Luczak M, Jagodziński PP, Skrzypczak J. Reduced  
 488 expression of HOXA10 in the midluteal endometrium from infertile women with minimal  
 489 endometriosis. *Biomed Pharmacother* 2010; 64:697–705.
- 490 [36] Fambrini M, Sorbi F, Bussani C, Cioni R, Sisti G, Andersson KL. Hypermethylation of  
 491 HOXA10 gene in mid-luteal endometrium from women with ovarian endometriomas.  
 492 *Acta Obstet Gynecol Scand* 2013; 92:1331–1334.
- 493 [37] Guo C, Ren F, Wang D, Li Y, Liu K, Liu S, Chen P. RUNX3 is inactivated by promoter  
 494 hypermethylation in malignant transformation of ovarian endometriosis. *Oncol Rep* 2014;  
 495 32:2580–8.
- 496 [38] Wang D, Chen Q, Zhang C, Ren F, Li T. DNA hypomethylation of the COX-2 gene  
 497 promoter is associated with up-regulation of its mRNA expression in eutopic

- 498 endometrium of endometriosis. *Eur J Med Res* 2012; 17:12.
- 499 [39] Szczepanska M, Wirstlein P, Skrzypczak J, Jagodzinski PP. Expression of HOXA11 in the  
500 mid-luteal endometrium from women with endometriosis-associated infertility. *Reprod*  
501 *Biol Endocrinol* 2012; 10:1.
- 502 [40] Naqvi H, Ilagan Y, Krikun G, Taylor HS. Altered Genome-Wide Methylation in  
503 Endometriosis. *Reprod Sci* 2014:1237–1243.
- 504 [41] Saare M, Modhukur V, Suhorutshenko M, Rajashekar B, Rekker K, Sõritsa D, Karro H,  
505 Soplepmann P, Sõritsa A, Lindgren CM, Rahmioglu N, Drong A, et al. The influence of  
506 menstrual cycle and endometriosis on endometrial methylome. *Clin Epigenetics* 2016; 8.
- 507 [42] Houshdaran S, Nezhat CR, Vo KC, Zelenko Z, Irwin JC, Giudice LC. Aberrant  
508 Endometrial DNA Methylome and Associated Gene Expression in Women with  
509 Endometriosis. *Biol Reprod* 2016; 95:93.
- 510 [43] Bulun S, Monsavaïs D, Pavone M, Dyson M, Xue Q, Attar E, Tokunaga H, Su E. Role of  
511 Estrogen Receptor- $\beta$  in Endometriosis. *Semin Reprod Med* 2012; 30:39–45.
- 512 [44] Zanatta A, Rocha AM, Carvalho FM, Pereira RMA, Taylor HS, Motta ELA, Baracat EC,  
513 Serafini PC. The role of the Hoxa10/HOXA10 gene in the etiology of endometriosis and  
514 its related infertility: a review. *J Assist Reprod Genet* 2010; 27:701–10.
- 515 [45] Xue Q, Zhou YF, Zhu SN, Bulun SE. Hypermethylation of the CpG island spanning from  
516 exon II to intron III is associated with steroidogenic factor 1 expression in stromal cells of  
517 endometriosis. *Reprod Sci* 2011; 18:1080–4.
- 518 [46] Xue Q, Xu Y, Yang H, Zhang L, Shang J, Zeng C, Yin P, Bulun SE. Methylation of a  
519 Novel CpG Island of Intron 1 Is Associated With Steroidogenic Factor 1 Expression in  
520 Endometriotic Stromal Cells. *Reprod Sci* 2014; 21:395–400.

- 521 [47] Xue Q, Lin Z, Yin P, Milad MP, Cheng Y-H, Confino E, Reierstad S, Bulun SE.  
 522 Transcriptional Activation of Steroidogenic Factor-1 by Hypomethylation of the 5' CpG  
 523 Island in Endometriosis. *J Clin Endocrinol Metab* 2007; 92:3261–3267.
- 524 [48] Izawa M, Taniguchi F, Uegaki T, Takai E, Iwabe T, Terakawa N, Harada T.  
 525 Demethylation of a nonpromoter cytosine-phosphate-guanine island in the aromatase gene  
 526 may cause the aberrant up-regulation in endometriotic tissues. *Fertil Steril* 2011; 95:33–  
 527 39.
- 528 [49] Wu M-H, Lin S-C, Hsiao K-Y, Tsai S-J. Hypoxia-inhibited dual-specificity phosphatase-2  
 529 expression in endometriotic cells regulates cyclooxygenase-2 expression. *J Pathol* 2011;  
 530 225:390–400.
- 531 [50] Dyson MT, Roqueiro D, Monsivais D, Ercan CM, Pavone ME, Brooks DC, Kakinuma T,  
 532 Ono M, Jafari N, Dai Y, Bulun SE. Genome-wide DNA methylation analysis predicts an  
 533 epigenetic switch for GATA factor expression in endometriosis. *PLoS Genet* 2014;  
 534 10:e1004158.
- 535 [51] Konno R, Yamada-Okabe H, Fujiwara H, Uchiide I, Shibahara H, Ohwada M, Ihara T,  
 536 Sugamata M, Suzuki M. Role of immunoreactions and mast cells in pathogenesis of  
 537 human endometriosis--morphologic study and gene expression analysis. *Hum Cell* 2003;  
 538 16:141–149.
- 539 [52] Yamagata Y, Nishino K, Takaki E, Sato S, Maekawa R, Nakai A, Sugino N. Genome-  
 540 wide DNA methylation profiling in cultured eutopic and ectopic endometrial stromal cells.  
 541 *PLoS One* 2014; 9:e83612.
- 542 [53] Kitawaki J, Noguchi T, Amatsu T, Maeda K, Tsukamoto K, Yamamoto T, Fushiki S,  
 543 Osawa Y, Honjo H. Expression of aromatase cytochrome P450 protein and messenger



- 544 ribonucleic acid in human endometriotic and adenomyotic tissues but not in normal  
545 endometrium. *Biol Reprod* 1997; 57:514–9.
- 546 [54] Xue Q, Zhou YF, Zhu SN, Bulun SE. Hypermethylation of the CpG island spanning from  
547 exon II to intron III is associated with steroidogenic factor 1 expression in stromal cells of  
548 endometriosis. *Reprod Sci* 2011; 18:1080–4.
- 549 [55] Fouquet B, Santulli P, Noel J-C, Misrahi M. Ovarian-like differentiation in eutopic and  
550 ectopic endometrioses with aberrant FSH receptor, INSL3 and GATA4/6 expression.  
551 *BBA Clin* 2016; 6:143–152.
- 552 [56] Ning Q, Li F, Wang L, Li H, Yao Y, Hu T, Sun Z. S100A4 amplifies TGF- $\beta$ -induced  
553 epithelial-mesenchymal transition in a pleural mesothelial cell line. *J Investig Med* 2018;  
554 66 (2):334–339.
- 555 [57] Yang Y-M, Yang W-X. Epithelial-to-mesenchymal transition in the development of  
556 endometriosis. *Oncotarget* 2017; 8:41679–41689.
- 557 [58] Neumann E, Riepl B, Knedla A, Lefèvre S, Tarner IH, Grifka J, Steinmeyer J,  
558 Schölmerich J, Gay S, Müller-Ladner U. Cell culture and passaging alters gene expression  
559 pattern and proliferation rate in rheumatoid arthritis synovial fibroblasts. *Arthritis Res*  
560 *Ther* 2010; 12:R83.
- 561 [59] Januszyk M, Rennert RC, Sorkin M, Maan ZN, Wong LK, Whittam AJ, Whitmore A,  
562 Duscher D, Gurtner GC. Evaluating the Effect of Cell Culture on Gene Expression in  
563 Primary Tissue Samples Using Microfluidic-Based Single Cell Transcriptional Analysis.  
564 *Microarrays (Basel, Switzerland)* 2015; 4:540–50.
- 565 [60] Bork S, Pfister S, Witt H, Horn P, Korn B, Ho AD, Wagner W. DNA methylation pattern  
566 changes upon long-term culture and aging of human mesenchymal stromal cells. *Aging*

- 567 Cell 2010; 9:54–63.
- 568 [61] Klemmt PAB, Carver JG, Kennedy SH, Koninckx PR, Mardon HJ. Stromal cells from  
569 endometriotic lesions and endometrium from women with endometriosis have reduced  
570 decidualization capacity. *Fertil Steril* 2006; 85:564–72.
- 571 [62] Classen-Linke I, Kusche M, Knauthe R, Beier HM. Establishment of a human endometrial  
572 cell culture system and characterization of its polarized hormone responsive epithelial  
573 cells. *Cell Tissue Res* 1997; 287:171–85.
- 574 [63] Pierro E, Minici F, Alesiani O, Miceli F, Proto C, Screpanti I, Mancuso S, Lanzone A.  
575 Stromal-epithelial interactions modulate estrogen responsiveness in normal human  
576 endometrium. *Biol Reprod* 2001; 64:831–8.
- 577 [64] Aghajanova L, Hamilton A, Kwintkiewicz J, Vo KC, Giudice LC. Steroidogenic enzyme  
578 and key decidualization marker dysregulation in endometrial stromal cells from women  
579 with versus without endometriosis. *Biol Reprod* 2009; 80:105–14.
- 580 [65] Chen J, Roan N. Isolation and Culture of Human Endometrial Epithelial Cells and Stromal  
581 Fibroblasts. *Bio Protoc* 2015; 5 (20).
- 582 [66] Masuda A, Katoh N, Nakabayashi K, Kato K, Sonoda K, Kitade M, Takeda S, Hata K,  
583 Tomikawa J. An improved method for isolation of epithelial and stromal cells from the  
584 human endometrium. *J Reprod Dev* 2016; 62:213–8.
- 585 [67] Kyo S, Nakamura M, Kiyono T, Maida Y, Kanaya T, Tanaka M, Yatabe N, Inoue M.  
586 Successful immortalization of endometrial glandular cells with normal structural and  
587 functional characteristics. *Am J Pathol* 2003; 163:2259–69.
- 588 [68] Sanchez AM, Vigano P, Somigliana E, Cioffi R, Panina-Bordignon P, Candiani M. The  
589 Endometriotic Tissue Lining the Internal Surface of Endometrioma: Hormonal, Genetic,

- Epigenetic Status, and Gene Expression Profile. *Reprod Sci* 2015; 22:391–401.
- [69] Liu H, McDowell TL, Hanson NE, Tang X, Fujimoto J, Rodriguez-Canales J. Laser Capture Microdissection for the Investigative Pathologist. *Vet Pathol* 2014; 51:257–269.
- [70] Dietrich D, Lesche R, Tetzner R, Krispin M, Dietrich J, Haedicke W, Schuster M, Kristiansen G. Analysis of DNA methylation of multiple genes in microdissected cells from formalin-fixed and paraffin-embedded tissues. *J Histochem Cytochem* 2009; 57:477–89.
- [71] Krjutskov K, Katayama S, Saare M, Vera-Rodriguez M, Lubenets D, Samuel K, Laisk-Podar T, Teder H, Einarsdottir E, Salumets A, Kere J, Krjutškov K, et al. Single-cell transcriptome analysis of endometrial tissue. *Hum Reprod* 2016; 31:844–53.
- [72] Rekker K, Saare M, Eriste E, Tasa T, Kukuškina V, Roost AM, Anderson K, Samuel K, Karro H, Salumets A, Peters M. High-throughput mRNA sequencing of stromal cells from endometriomas and endometrium. *Reproduction* 2017; 154:93–100.
- [73] Logan PC, Yango P, Tran ND. Endometrial Stromal and Epithelial Cells Exhibit Unique Aberrant Molecular Defects in Patients With Endometriosis. *Reprod Sci* 2017; 1:140–159.
- [74] Sumathi VP, McCluggage WG. CD10 is useful in demonstrating endometrial stroma at ectopic sites and in confirming a diagnosis of endometriosis. *J Clin Pathol* 2002; 55:391–392.
- [75] Takao Y, Fujiwara H, Yamada S, Hirano T, Maeda M, Fujii S, Ueda M. CD9 is expressed on the cell surface of human granulosa cells and associated with integrin alpha6beta1. *Mol Hum Reprod* 1999; 5:303–10.
- [76] Teschendorff AE, Zheng SC. Cell-type deconvolution in epigenome-wide association studies: a review and recommendations. *Epigenomics* 2017; 9:757–768.

- 613 [77] Zou J, Lippert C, Heckerman D, Aryee M, Listgarten J. Epigenome-wide association  
614 studies without the need for cell-type composition. *Nat Methods* 2014; 11:309–311.
- 615 [78] Houseman EA, Molitor J, Marsit CJ. Reference-free cell mixture adjustments in analysis  
616 of DNA methylation data. *Bioinformatics* 2014; 30:1431–1439.
- 617 [79] Rahmani E, Zaitlen N, Baran Y, Eng C, Hu D, Galanter J, Oh S, Burchard EG, Eskin E,  
618 Zou J, Halperin E. Sparse PCA corrects for cell type heterogeneity in epigenome-wide  
619 association studies. *Nat Methods* 2016; 13:443–445.
- 620 [80] McGregor K, Bernatsky S, Colmegna I, Hudson M, Pastinen T, Labbe A, Greenwood  
621 CMT. An evaluation of methods correcting for cell-type heterogeneity in DNA  
622 methylation studies. *Genome Biol* 2016; 17:84.

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**Figure legend**

**Figure 1.** The impact of tissue heterogeneity on endometriosis DNA methylation studies. A. Peritoneal lesions and endometriomas contain only a small fraction of endometrium-specific cells (brown colour in endometrioma and peritoneal lesion histological section indicates CD10<sup>+</sup> endometrial stromal cells that surround the endometrial epithelial glandular structure) surrounded by other cell-types. This may lead to a heterogeneous DNA methylation signature as the same CpG sites may have a cell-type specific methylation status. B. Stromal cells isolated from endometrioma and endometrium are with low cellular heterogeneity (immunofluorescence staining of cultured stromal cells using antibody against CD10) and the obtained DNA methylation signature is characteristic to a homogeneous cell population.