Harmonization of Clinical and Laboratory Data to Improve Biomarker Discovery in Endometriosis: WERF EPHect

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Abstract Endometriosis is a heterogeneous condition in terms of surgical characterization of the disease and nonsurgical symptomatic and non-symptomatic characteristics of the woman. Many centers across the world conduct research into endometriosis independently from each other, using different standard operating protocols (SOPs) for collection of biological samples and different questionnaires for capturing clinical and surgical phenotypes. The aim of the World Endometriosis Research Foundation (WERF) Endometriosis Phenome and Biobanking Harmonisation Project (EPHect) is to standardize globally the collection of samples

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and phenotypes across centers, allowing for more effective large-scale international collaborative research of the condition. To achieve this goal, two workshops were conducted in 2013, bringing together 54 clinical, academic, and industry leaders in endometriosis research from 16 countries. SOPs and questionnaires from the contributing centers were systematically compared, and available literature evidence, along with consultation from laboratory experts, was taken into consideration to reach consensus SOPs and questionnaires. After several global revisions, two-level *standard recommended* and *minimum required* (1) forms for collection of surgical phenotypes; (2) questionnaire for collection of clinical phenotypes; (3) SOPs for blood, saliva, urine, endometrial/peritoneal fluid, menstrual effluent; and (4) SOPs for ectopic and eutopic endometrium, peritoneum, and myometrium were published. These instruments will be updated regularly based on feedback from investigators, and current versions are available through http://endometriosisfoundation.org/ephect.

Keywords Endometriosis • Harmonization • Standardization • Biobanking • Phenotypic data collection • Pelvic pain • Infertility • Biospecimen collection • Collaboration • SOPs

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Introduction

Endometriosis is a heterogeneous condition with respect to its natural history, disease burden, extent of inflammation, state of progression, and phenotypic presentation of lesions and symptoms. The variability of patient "types" included in endometriosis research studies is determined by both (1) the surgical characterization of the extent of disease during laparoscopy and (2) symptomatology (onset, duration, extent and severity of symptoms, comorbidity) and other non-symptomatic phenotypes such as anthropometric characteristics, ethnicity, and reproductive and demographic factors. Until recently, no consensus existed on even the minimum surgical information that should be collected to perform clinical and basic science studies for endometriosis. This is reflected in the varying and conflicting results in biomarker studies for endometriosis [1–3]. Currently available data sets on endometriosis cases and controls typically (1) lack surgical and symptomatic phenotype detail combined with biological sample information, (2) are not sufficiently consistent in terms of the type of data collected and protocols used to allow the collaborative exploration of the abovementioned associations, or (3) are limited by sample size.

In terms of the surgical data collection, while there is consensus that laparoscopy remains the gold standard for a definitive diagnosis of endometriosis [4–6], investigators are advised to take full advantage of the diagnostic aspect of the procedure by collecting more standardized detailed information during laparoscopic surgery and optimize the characterization of the surgical phenotype. In addition, for nonsurgical symptom or non-symptom-related characteristics, the use of standardized detailed questionnaires should optimize characterization of the different patient "types." Moreover, to study the phenotypic variation successfully, studies need to include sufficient numbers of patients to allow for the detection of differences between subphenotype groups with adequate statistical power. Collaboration and pooling of individual participant data across research centers can enable much larger sample sizes, allowing for subgroup analyses and meaningful comparison between different patient populations in endometriosis research. Indeed, successful risk factor and sub-phenotype investigations among many centers have been demonstrated by large consortia across an array of disease outcomes [7–13].

In addition, many centers worldwide have been collecting biological fluid and tissue samples from women with and without endometriosis, with the aim to identify potential diagnostic biomarkers and novel drug targets for the disease [14]. Molecular profiles obtained toward these goals include, but are not limited to, changes at the deoxyribonucleic acid (DNA), ribonucleic acid (RNA), protein, and metabolite levels detected in various bodily fluids and tissues. However, variability in specimen collection, processing, and storage methods can act as a considerable source of bias and measurement error, obscuring detection of disease-related molecular perturbations [15, 16].

Standard operating procedures (SOPs) and recommendations for blood collection in reproductive biology research have been published [1, 17], but—until recently—there were none for other fluid specimens such as urine, saliva, or peritoneal and endometrial fluid. Likewise for eutopic endometrium collection, the

University of California, San Francisco, NIH Human Endometrial Tissue and DNA Bank (http://obgyn.ucsf.edu/crs/tissue_bank/) published well-described SOPs specifically to allow collaborative research [17], but none existed for the other endometriosis-related tissue specimens such as ectopic endometrium, myometrium, and peritoneum. Standardized collection of biospecimens across centers using internationally agreed-on SOPs—based on existing scientific evidence and consensus—is likely to reduce variability and facilitate comparability of results and enhance the detection of endometriosis biomarker relationships through multicenter collaborative studies. Successful collaborative investigation of fluid and tissue markers has been well established in the investigation of other disease outcomes [18–24].

The objective of the WERF Endometriosis Phenome and Biobanking Harmonisation Project (EPHect) was to develop a consensus on standardization and harmonization of phenotypic surgical/clinical data and biological sample collection methods in endometriosis research. Through a series of workshops and global consultations involving 54 clinical, academic, and industry leaders in endometriosis research from 16 countries, a set of standardized surgical and clinical data collection tools and SOPs of biospecimen collections were developed [25–28]. These instruments facilitate—for the first time—large-scale internationally collaborative, longitudinal, epidemiologically robust, translational biomarker and treatment target discovery research in endometriosis [14, 29].

Here, we have summarized the EPHect consensus on:

1. Standardized surgical data and sample collection in women undergoing laparoscopy
2. Standardized collection of nonsurgical/clinical and epidemiological phenotypic data through patient-administered questionnaires
3. Standardized SOPs for biological fluid
4. Tissue collection, processing, and long-term storage to enable cellular, genetic, molecular, proteomic, metabonomic, and transcriptomic studies

We acknowledge that there are likely to be differences in resources and logistics among centers that influence feasibility of adherence to some of the strictest standards of data collection and SOP implementation. Therefore, WERF EPHect developed two-tiered data collection instruments and biospecimen SOPs: a standard recommended version and a minimum required version.

Materials and Methods

Setting

Two workshops were conducted in March and July 2013, bringing together 54 clinical, academic, and industry leaders in endometriosis research from 16 countries on five continents, to develop and reach consensus on evidence-based

phenotype collection and SOP guidelines. During workshop I, four areas of standardization and harmonization were defined: (1) surgical phenotyping, (2) nonsurgical clinical/epidemiologic phenotyping, (3) fluid sample, and (4) tissue sample collection, processing, and storage protocols for molecular and genetic analysis. The workshop was followed by e-mail consultation round including open invitations sent to all 54 WERF EPHect collaborators, asking them to review the data collection tools and SOPs under development and to participate in workshop II. During workshop II, the data collection tools and SOPs were presented to participants together with a summary of reviews obtained through e-mail consultations and literature-based evidence. Draft consensus data collection tools and SOPs were subsequently reviewed during several rounds of expert review by the WERF EPHect working group (Fig. 1).

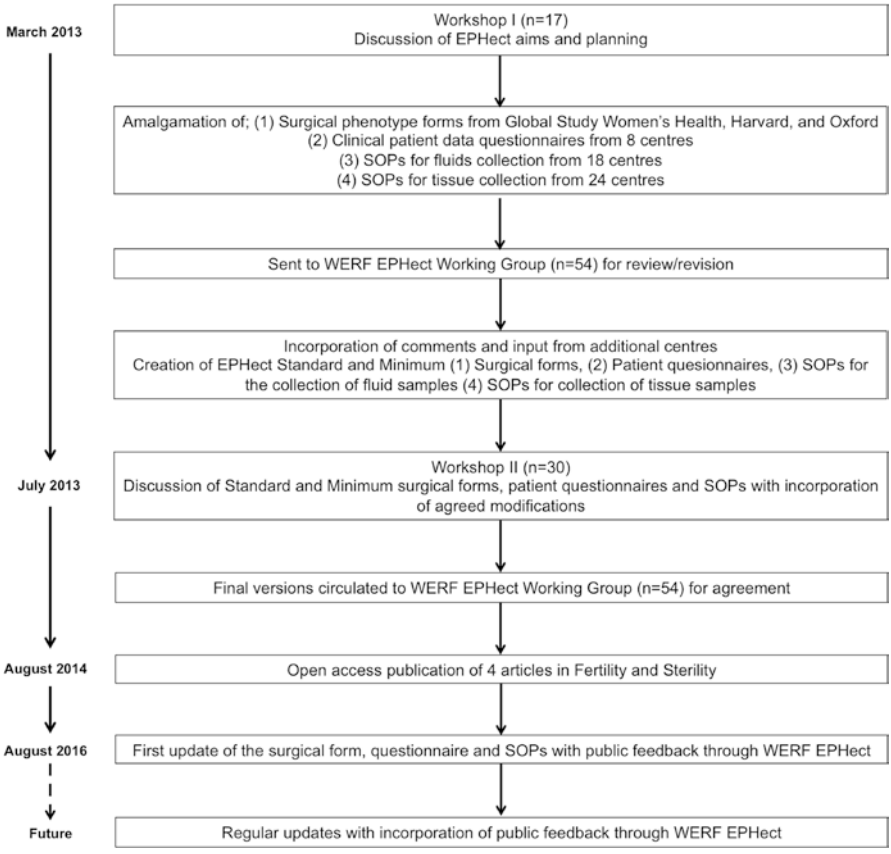

Fig. 1 Flow diagram depicting the WERF EPHect development and consensus process (Adapted from [25–28])

Harmonization Procedure for Surgical Phenotypic Data

The first draft of the surgical data collection instrument was based on a postsurgical scoring sheet, originally developed as part of the WERF Global Study of Women's Health [30, 31], which had recently been extended and piloted in the Boston Center for Endometriosis and the Endometriosis CaRe Centre Oxford. The scoring sheet contained general and gynecological information about the patient, the procedure, extent of disease, and the location and type of endometriotic lesion, along with existing tools for disease classification such as the revised AFS [32] and EFI [33]. This form was discussed and extended during several review rounds by experts in the field of endometriosis using surgical data collection tools that were in use at their centers.

Adaptation of the standard recommended version of the surgical data collection instrument (SSF) will be of central importance for current and future advancement in understanding the biology of disease and investigation of the effects of treatment on symptoms and disease recurrence. The minimum required version (MSF) is the basic requirement for more limited research studies in settings where completion of the standard instrument is logistically impossible. Both forms are designed for surgery involving women with confirmed endometriosis and symptomatic or asymptomatic women free from endometriosis (http://endometriosisfoundation.org/ephect/).

Harmonization Procedure for Nonsurgical Phenotypic Data

The initial development of the nonsurgical patient questionnaire was based on questionnaire tools provided by eight centers worldwide that have collected nonsurgical information from endometriosis cases and controls on a large scale (criterion, publication on >100 cases); all provided the patient questionnaires used. These questionnaires were reviewed, and key topics were identified for inclusion in the draft consensus endometriosis patient questionnaire (EPQ), including pelvic pain, infertility and reproductive history, menstrual history and hormone use, medical and surgical history, medication use, and personal information. A subsequent e-mail consultation was conducted including all 54 EPHect collaborators, asking them to review the EPQ.

An extensive literature search was conducted in PubMed for English language publications describing associations between the key topics included in the EPQ and endometriosis. Rigorous review of the phrasing and temporality of each question on the EPQ was performed by the clinical and epidemiologic experts in the WERF EPHect working group. Importantly, the EPQ development focused on selecting questions and rating scales that are validated in the literature. In addition, most questions were piloted by patients and volunteers in the centers contributing the questions, and all questions were reviewed by the workshop participants. During workshop II, the questionnaire was presented to participants together with

a summary of reviews obtained through e-mail consultation, and a consensus was obtained on the final content and format of the questionnaire. All participants in the consultation were asked to decide which information in the EPQ should be collected as a minimum (EPQ-M) requirement and which would be recommended as standard (EPQ-S), to reach the consensus on this division.

The development of the EPQ focused on information that was considered by the WERF EPHect working group to be universally important to endometriosis centers in characterizing patients by their spectrum of symptoms. We did not include many potentially important exposures that may be associated with endometriosis etiologically and that may be of specific interest to some centers but were not considered crucial for patient characterization. These include, for example, nevi and freckles, sun exposure, in utero exposures, and others exposures [34]. Investigators adopting the EPQ are encouraged to add any additional questions they would like to further their own scientific aims and state these adaptations in resulting publications.

Harmonization Procedure for Fluid and Tissue Biospecimen Collection

Fluid Biospecimen SOPs

A total of 18 centers worldwide were identified that collect biologic fluid samples from endometriosis cases and controls on a large scale (criterion, publication on >100 cases); all provided SOPs for sample collection, processing, and storage. Six fluid sample types were collected by the centers (blood, urine, saliva, peritoneal fluid, endometrial fluid, and menstrual fluid). In addition to the information provided by the 18 centers, publicly available SOPs were searched from general large-scale biobanking efforts (e.g., UK Biobank) and large biorepositories (International Society for Biological and Environmental Repositories [ISBER], the NCI Biorepositories and Biospecimen Research Branch [NCI-BBRB], and the Australian Biospecimen Network [ABRN]). A systematic literature search was conducted in PubMed for English language publications describing crucial steps in SOPs, using the following search terms: “standard operating procedure” with “endometriosis,” “blood,” “urine,” “endometrial fluid,” “peritoneal fluid,” “menstrual effluent,” “fluid samples,” “best practice,” or “biobank.”

Tissue Biospecimen SOPs

A total of 24 centers were identified worldwide that collect tissues from endometriosis case and control subjects on a large scale (publication on >100 cases); all provided SOPs for sample collection, processing, and storage. Four tissue types (ectopic endometrium, eutopic endometrium, myometrium, and peritoneum) were collected by these centers. In addition to the information provided by the 24 centers,

publicly available SOPs were searched from general large-scale biobanking efforts (UK Biobank) and large biorepositories (ISBER, NCI-BBRB, ABRN), and a systematic literature search was conducted in PubMed for English language publications describing crucial steps in SOPs, with the use of the search terms: "standard operating procedure" with "endometriosis," "tissues," "endometrium," "myometrium," "peritoneum," "best practice," or "biobank."

On the basis of this information, we compiled draft consensus fluid and tissue SOPs, identifying steps that varied between center-specific SOPs, but for which little or no evidence could be obtained. Prior to workshop II, consensus documents and associated evidence and queries were distributed to the WERF EPHect working group. During workshop II and a separate e-mail consultation process, the final consensus SOPs were reviewed and agreed upon.

WERF EPHect strongly advises standard recommended collection SOPs to be adopted when possible, as they will yield results that are least prone to variation and degradation of the samples; the minimum required SOP steps are offered to provide the fundamentals for standardization that need to be adhered to as an absolute minimum requirement given unavoidable logistical and budgetary circumstances. It is important to note that publications of results generated using samples collected following the EPHect SOPs need to state explicitly, which EPHect procedures were used and any alterations made to them.

When collecting biologic samples for research purposes, additional data items need to be collected to allow interpretation of results from the samples, such as recent medication use by the participant and her menstrual cycle phase at the time of sample collection. For this purpose, the WERF EPHect working group developed a consensus biospecimen form to be completed at each sample collection event.

Results: Standardization of Surgical Phenotypic Data

The rationale behind the development of the WERF EPHect surgical data collection forms (the standard [recommended] surgical form [SSF] and minimum [required] surgical form [MSF]) is described below.

EPHect SSF

The SSF is divided into two parts. The first part includes detailed information about clinical covariates including the current menstrual cycle, current hormone treatment, and history of previous endometriosis surgery, as well as any imaging findings before the procedure. The second part is on intraoperative findings, including the type and duration of the procedure and the extent, exact location, and color of endometriotic lesions, with a particular focus on the size of endometrioma and endometriotic nodules. It allows for an exact description of tissue biopsies (see

section "Biological Sample Collection"), including their location and appearance, and surgical treatment of lesions.

For reference on interpretation of appearance of lesions, pictures of representative endometriotic lesions are given in Becker et al. [25].

Pilot work has shown that after an initial brief learning period, the SSF takes about 1–3 min to complete, depending on the extent of disease and sample taking to be recorded.

EPHect MSF

The sole aim of developing the MSF was to identify the essential, basic, surgical information that a surgeon under considerable time constraints would be able to complete accurately and consistently after surgery. The MSF will enable a group to start gathering relevant surgical phenotypic information where such information was not systematically collected before. The MSF is also divided into two parts, asking about clinical covariates and intraoperative findings but in less detail.

Video/Photo Documentation

To evaluate the presence or absence of endometriotic lesions, adhesions, and cysts, it is vital to systematically and meticulously search the entire pelvis and abdominal cavity with a laparoscope. Where permitted, video recording of pelvic exploration and surgical procedures is the recommended standard [35]. In addition, photo documentation is strongly recommended to provide an objective record of the reported data (including for research purposes). In addition to exploring the clinical and molecular phenotype of the individual lesions, it may be that unique and critical information can be discovered from the colony/cluster/microenvironment of lesions proximal to each other. These phenotypic details can only be documented and quantified via video and/or photographic documentation. Becker et al. show the photo documentation to be collected as the standard recommendation by EPHect [25].

Biological Sample Collection

Biological samples relevant to endometriosis research could be collected during laparoscopic surgery. The results on detailed WERF EPHect SOPs for collection, processing, and long-term storage of these samples are described in the harmonization of fluid and tissue biospecimen collection sections [26, 27].

WERF EPHect recommends the collection of samples in a prespecified order and with optimal SOPs implemented from the moment of surgical extraction of the

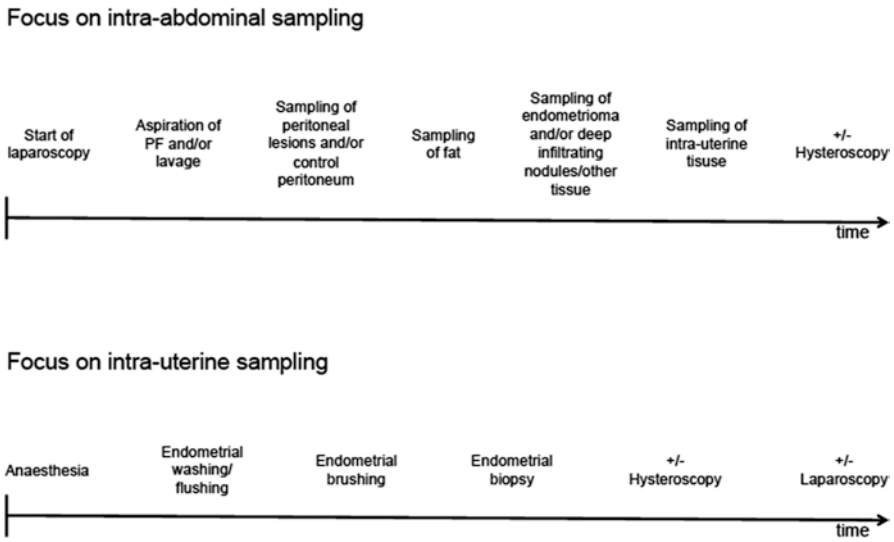

Fig. 2 Suggested timeline for biological sample collection depending on research question (From [25])

sample. Sampling should be performed as early as possible to diminish a possible impact of anesthetic drugs and minimize contamination by blood or distension fluids [36–40]. Provided it is clinically justifiable, the order of samples collected is prioritized by the research question as depicted in Fig. 2. For example, if intra-abdominal sampling (peritoneum, peritoneal fluid, endometriotic disease) is the main focus, it is recommended to perform laparoscopy before hysteroscopy to avoid contamination from hysteroscopic fluid. However, for clinical purposes, it may be necessary to perform hysteroscopy first. Nevertheless, it is important to record the order of surgical procedures and the type of hysteroscopic fluid used in the SSF.

If peritoneal fluid is collected, this should be the first intra-abdominal sample collected to reduce the risk of contamination with blood, cyst fluid, or tissue. The volume of peritoneal fluid is influenced by menstrual phase [41]. If no or very limited peritoneal fluid is available, then a lavage with sterile saline (10 mL) over pelvic organs and walls is the standard recommendation (see section "Peritoneal Fluid Stability, Processing, and Storage" section in "Harmonization of Fluid Biospecimen Collections").

Next is the collection of endometriotic peritoneal lesions from endometriosis patients and normal peritoneal tissue from the healthy control patients. Owing to the anatomic location and possible surgical complexity, endometriomas and deep infiltrating nodules are commonly the last samples to be collected. It is a standard recommendation to record the temperature of the CO_2 entering the abdomen and the presence or absence of a gas humidifier on the SSF.

If the main research focus is on uterine sampling (eutopic endometrium and myometrium), then it is preferable to begin with the endometrial biopsy to reduce the potential effect of the anesthetic drugs or potential endocrine or paracrine influences on the sample (Fig. 2). It is the standard recommendation to collect endometrial samples before insertion of a uterine manipulator as this is likely to affect the sample quality. The type and date of any prior intrauterine procedures, such as hysteroscopy or endometrial biopsy, should be recorded as part of the MSF.

If surgically feasible, the use of thermal energy should be avoided for all tissue collections, as these may impact the histological interpretation of the tissue [42] and the expression of biomolecules. If thermal energy is required, then it is recommended to use laser or plasma jet with as little energy as clinically possible and to leave a safety margin of 5 mm.

Results: Standardization of Nonsurgical Phenotypic Data

The rationale behind the development of standard and minimum versions of the WERF EPHect endometriosis patient questionnaire (EPQ-S and EPQ-M) is described below. The standard questionnaire (EPQ-S) is a 30-page self-administered questionnaire on comprehensive phenotypic description of endometriosis symptomatology, menstrual and reproductive history, various lifestyle factors, and medication use of the subjects. In the minimum patient questionnaire (EPQ-M), the symptoms and characteristics pertaining across the life course are excluded even though they are crucial to characterize women with and without endometriosis. Pilot studies have shown that the standard EPQ-S takes 25–40 min to complete. In settings when the completion of EPQ-S will impact study recruitment because of its length, EPQ-M can be used instead.

Pain

Most common pain symptoms experienced by endometriosis patients are dysmenorrhea, noncyclical pelvic pain, dyspareunia, and dyschezia. The relationship between endometriosis and these pain symptoms is complex with little correlation between extent of disease and severity of pain experienced by the patient [43, 44].

Recommendations have been published for standard endometriosis-associated pain data collection techniques [45]. Using these guidelines, for the EPQ, the pain intensity is measured on an 11-point numerical scale (NRS), 0 being no pain and 10 being the worst imaginable pain. On the EPQ, pain effect is captured with the short form McGill Pain Questionnaire (SF-MPQ). However, we recommend as standard the use of the most recent SH-MPQ-2, as ratings are given on an 11-point scale, similar to measures of pain intensity, and seven additional questions allow for

calculation of four separate domains (continuous pain, intermittent pain, neuropathic pain, and affective) and a total score as opposed to the original version which only calculates two domains (sensory and affective) and a total score [46]. SH-MPQ-2 requires each center to sign a user agreement form, which is why it was not included in the EPQ.

Of all the cognitive and psychological covariates commonly measured in experimental and clinical pain studies, pain catastrophizing [47] is identified as the most robust measure associated with indices of pain sensitivity, clinical outcomes, and behavioral expressions of pain [48]. Therefore, a pain catastrophizing scale is included in the EPQ.

Depression, Anxiety, and Health-Related Quality of Life

Questions on the psychological state and health-related quality of life in a symptom-based questionnaire are important as these factors may affect responses related to symptomatology. The validated generic health status measures, such as the Endometriosis Health Profile (EHP-30) questionnaire [49] or the Short-Form Health Status Survey (SF-36) [50], were not included in the EPQ since their use requires registration and/or payment from the individual centers. Additionally, validated depression and anxiety scales can be helpful for patient stratification such as the Beck Depression Inventory (BDI) [51] and the State-Trait Anxiety Inventory (STAI) [52]. WERF EPHect recommends that individual sites consider including these additional scales when adopting the EPQ at their centers.

Menstrual History and Hormone Use

Age at menarche and menstrual cycle characteristics in the last 3 months are captured in detail as they have been robustly associated with endometriosis [53–57], are likely to influence symptom reporting, and are crucial for interpretation of molecular assays. Furthermore, lifetime menstrual cycle characteristics and their change over time may be important in understanding the etiology of endometriosis.

For capturing regularity, frequency, duration, and heaviness of menstrual flow, the International Federation of Gynecology and Obstetrics (FIGO) guidelines [58] were adapted in the EPQ. Menstrual flow is classified as spotting, light, moderate, and heavy using previously validated menstrual pictogram [59].

A complete history of hormone use is captured in the questionnaire, as this information is crucial for interpretation of the reported symptomatology. Furthermore, long-term and recent hormone use can affect biomolecule profiles [60–62].

Infertility and Reproductive History

Fertility impairments such as delay in conception and infertility are associated with endometriosis [4], though the relationship between causality and diagnostic bias between these outcomes is unknown. Infertility is assessed by the longest time (>6 months) a study participant has tried to become pregnant without success and any test she might have had to find the cause of infertility. The standard definition of infertility is 12 months of regular unprotected intercourse without achieving a clinical pregnancy [63], and this definition can be derived from data collected within the EPQ. However, a 6-month screen cutoff was selected here since older women may seek medical intervention before reaching the 12-month period.

A detailed pregnancy history is also captured by the EPQ, including age at the start of each pregnancy, type of fertility treatment used for each pregnancy, and pregnancy outcome. Further details for live births include whether the pregnancy was multiple gestation, the delivery method, and pregnancy complications. Retrospective studies suggest that women with endometriosis have higher rates of maternal complications, fetal problems, and miscarriage [64–68], although these associations need further confirmations.

Medical and Surgical History

Comorbidity is an important confounding factor in assessing the extent and severity of symptoms. In the EPQ, women are asked if they have ever been diagnosed and age of diagnosis with a list of ~30 medical conditions, including cancer, gynecologic disease, pain syndromes, and autoimmune diseases [69–72]. Surgical history including age at surgery, type, and indications is also enquired that can be etiologically related to pelvic pain symptoms and impact on symptom reporting.

In addition women are asked about recent bowel and urinary symptoms. For the bowel symptoms that are common in women with endometriosis, questions from the Rome III criteria irritable bowel syndrome module are included [73].

A diagnostic history for endometriosis is questioned in detail including age at first symptoms, age and method of diagnosis, and any prior surgical treatments. Also, family history of endometriosis or chronic pelvic pain is obtained, recognizing that accuracy of diagnosis is varied across generations.

Medication Use

Collection of recent medication use is important in biomarker studies since some drugs can interact with the biomarkers, clouding the results. Recent medication use is not captured in detail in the EPQ; however, in the biospecimen form that is

required to be completed along with fluid or tissue biospecimen collections, including a detailed section on medication use in the past 30 days and 48 h before biospecimen collection. The questions on medication use on the EPQ are to capture medication that could influence how women respond to questions. For example, medication for chronic pain or inflammatory conditions or for other symptoms including depression or anxiety may affect pain reporting.

Personal Information

Demographic data, including age, race/ethnicity, major ancestry, and highest level of education attained, that are required for interpretation of any epidemiological study are collected on EPQ.

Anthropometric measurements such as body mass index (BMI; current weight and height), most and least weighed since age 18, somatotype by age range [74], and body shape by age range [75] are recorded. Current BMI has been shown to be inversely associated with endometriosis [76] and validly measured by self-reported questionnaires [77–79]. Two questions on hair and eye color, previously associated with endometriosis [80–84], are also included. Lastly, basic questions on smoking, alcohol use [85], and exercise are included.

Results: Harmonization of Fluid Biospecimen Collection

The rationale behind the development of the WERF EPHect SOPs (standard recommended and minimum required) and the biospecimen form for recording of associated data for collection, processing, and long-term storage of blood and its derivatives (serum, plasma, and red/white blood cells), urine, saliva, peritoneal fluid, endometrial fluid, and menstrual effluent is given below.

Blood

Blood tissue is stored after separation into serum, plasma, and red/white blood cells for widest future use possibilities. However, blood tissue has a complex mix of molecules that do not only reflect changes relevant to the disease.

1. *Timing and conditions of sample collection*

   Dependent on the time of the day, the blood sample collected will have varying levels of various biomolecules due to physiological state, circadian rhythms, fasting status, or other factors that could result in changes in the endogenous concentrations of these. Therefore, ideally blood samples should be collected

after a 10-h fast [86]. Secondly, if samples are collected on the day of diagnostic surgery for endometriosis, they should be collected prior to induction of anesthesia, as these drugs can have an effect on the biomolecules of interest.

2. *Anticoagulants and clot accelerators*

   The type of anticoagulant used in the blood collection tubes determines how the sample can be used [87]. Particular anticoagulants are recommended for certain analytical purposes; therefore, selection of the appropriate anticoagulant for the assay of interest is crucial [88, 89]. EDTA tubes are often the most preferred type as they are suitable for wide variety of DNA and protein-based assays [87].

   If interested in storing the serum component of blood tissue, blood needs to be clotted, and the supernatant is the serum which can be separated with ease. Clots form very slowly in untreated tubes; however, there are serum separator tubes with clot accelerators that can speed up the process. Serum samples are suitable for most clinical biochemistry and metabolomics analyses, but they are not optimal for other assays such as proteomics due to clot-related peptides that contaminate the sample [90, 91].

3. *Sample stability between collection, and processing/storage*

   The time lapse and temperature conditions between sample collection and processing/storage are crucial factors affecting the stability of biomolecules in samples. In general, keeping samples at 4 °C from collection till storage minimizes enzymatic degradation of many biomolecules [92]. DNA is one of the most stable biomolecules [92], while RNA degrades within the first half hour of sample collection [17].

   For most uses, therefore, the blood samples should be processed and stored as soon as possible (within 2 h) or at most within 4 h [93, 94]. If there is a longer delay in processing, pilot studies are required to test the stability of the biomolecule of interest. For sensitivity biomolecules such as RNA, the integrity can be maintained by immediate addition of commercially available inhibitors of RNase enzymes. However, it should be noted that the addition of these RNase inhibitors compromises the utility of the sample for other assays and they can be costly in large-scale studies [87].

4. *Processing*

   Centrifugation is performed to separate blood into its components, and we suggest centrifugation at 2500 × g for 10 min, based on the typical parameter values observed in the contributing WERF EPHect centers and in the UK Biobank. Secondly, we recommend cooled (4 °C) centrifugation as standard to avoid effect of temperature on stability of the biomolecules.

5. *Long-term storage*

   The number and volume of the sample aliquots created should be a balance between minimizing future freeze-thaw cycles and use of freezer space. Repeated freeze-thaw cycles are detrimental to the stability of biomolecules in the samples [95, 96]. The samples should be stored as a minimum requirement in −80 °C mechanical freezers for long-term storage. Liquid nitrogen (LN_2) freezers are colder and have less temperature fluctuations and are recommended for standard long-term sample storage.

Urine

Urine samples are widely used in metabolomics and proteomic studies [93, 97, 98] because of its easy, noninvasive collection in large quantities [99]. However, many other molecules are excreted in the urine along with molecules of interest to the disease. It is vital to measure and adjust the molecules of interest to creatinine levels in sample to determine the concentration of the sample, as this varies substantially within individuals over time [100].

1. *Sample collection*
   Adapting a "clean catch" protocol for sample collection is important to reduce the incidence of microbial contamination of the samples. The timing of the sample collection for urine is complex as each urine sample reflects what was metabolized and excreted since the previous void. The most comprehensive approach could be to collect all urine voided over a 24-h period; however, this may not be feasible for most of the studies. Therefore, a first morning void sample can be collected as an alternative unless the participant voided during the night [101] and is better than a "spot urine" sample collected at a random time during the day [87, 99].
2. *Sample stability, processing, and storage*
   The standard recommendation is to maintain the urine sample at 4 °C until processing/storage to reduce the effects of possible enzymatic activity and store within 2 h of collection. If first morning void urine samples are collected, the participant should keep the collected sample in the refrigerator and transport the sample to the clinic on ice. Long-term storage of urine samples should ideally be in LN_2 freezers or in −80 °C freezers (see blood storage section).

Saliva

Saliva samples are most often used for DNA-based analysis when taking blood tissue from the participant is not desirable [102]. Other biomolecules such as hormones can also be measured in saliva; however, since they are found in only their free form, their concentrations are relatively low [103].

1. *Sample collection*
   Saliva samples can be collected with various methods including, "swish and spit," saliva collection kits for DNA and swabs. The "swish and spit" method or the Oragene® kits are recommended as standard in WERF EPHect as they provide the best DNA quality and yield [104–106]. For other biomolecule measurements, the "passive drool" method for sample collection is recommended as standard since other methods stimulate saliva production can alter hormone levels [103]. Furthermore, actively spitting tightens muscles and may affect the

flow rate and concentration of proteins in saliva [107, 108]. In terms of amount of sample collected, EPHect is recommending 2 mL as standard and 1 mL as the minimum requirement [109]. Timing of saliva collection is important if interested in measuring stress-related biomolecules [99]; therefore, recording time/date information is critical. Lastly, on the biospecimen form, it is important to record when the participant last brushed their teeth, chewed gum, smoked, or consumed alcohol, spicy food, or fishy food within the last 24 h, as these can affect sample quality.

2. *Sample stability, processing, and storage*

   Some salivary hormones are relatively stable in samples kept at room temperature for up to 1 week, although commination with mold can be problematic. Therefore, we recommend keeping samples chilled (4 °C) [110, 111]. For DNA extraction using commercial kits, the product instructions should be followed. Long-term storage should be in −80 °C freezers as a minimum requirement or in LN_2 freezers per standard (see blood storage section).

Peritoneal Fluid

1. *Sample collection*

   Peritoneal fluid is present in the peritoneal cavity, and its specific microenvironment is investigated for roles of various constituent biomolecules in relation to endometriosis [112–114].

2. *Sample stability, processing, and storage*

   Peritoneal fluid is aspirated using a syringe or suction device during laparoscopy, after entry into the pelvic cavity [25]. If no or very limited fluid is found, a lavage method can be used to wash the peritoneal surfaces with 10 mL sterile saline solution. This peritoneal lavage fluid (PLF) can be processed as peritoneal fluid, but the supernatant should be regarded with caution as molecular profiles may vary depending on the collection method used. Pilot studies are needed to compare the peritoneal microenvironments when sampling is performed using these two different methods. On the biospecimen form, the collection method and cycle phase should be recorded as they may affect the concentration of the biomolecules measured [114].

Endometrial Fluid and Menstrual Effluent

Endometrial fluid is found in the endometrial cavity in the uterus [115, 116] and reflects its specific microenvironment. Menstrual effluent is used for investigation of molecules in menstruation/endometrium-related processes such as angiogenesis and endometrial repair [117].

1. *Sample collection*
   Endometrial fluid is recommended to be collected without administration of any premedication or anesthetics using an embryo-transfer catheter connected to a syringe [25, 116]. If very limited fluid is found, a uterine lavage can be performed through slow infusion and withdrawal of 4 mL sterile saline solution into the uterine cavity [118]. This uterine lavage fluid (ULF) can be processed as endometrial fluid, but the supernatant from ULF should be regarded with caution. On the biospecimen form, the collection method and cycle phase (in menstrual phase this sample should not be collected) should be recorded as they may affect the concentration of the biomolecules measured [119]. Menstrual effluent is collected during menstrual phase with a diaphragm or mixing cannula [117].
2. *Sample stability, processing, and storage*
   The endometrial samples should be kept cool (4 °C) during processing and supernatant and pellet stored separately. If volume of the sample is not large enough for centrifugation, i.e., collected with embryo-transfer cannula, the cannula can be snap frozen immediately in LN_2. For long-term storage, samples per standard should be used in LN_2 freezers (see section "Blood Storage").

Results: Harmonization of Tissue Biospecimen Collection

The rationale behind the development of the EPHect SOPs (standard recommended and minimum required) for collection, processing, and long-term storage of ectopic, eutopic endometrium, myometrium, and peritoneum samples is given below. The collection methods for these tissues are distinct from each other; however, many aspects related to processing and storage are similar.

Methods of Collection

1. *Ectopic endometrium*
   Ectopic endometrium is excised using cold scissors/scalpels, electrosurgery, harmonic scalpel, or laser [25]. The presence of stromal and glandular epithelial cells should be verified histologically by an experience pathologist. Pathologic analysis of the tissues accrued before freezing or release for research needs to document the histologic characteristics of the tissues, and histology slides should be prepared in a cryostat at low temperatures to maintain the integrity of the tissue. Ectopic endometrium can be snap frozen in LN_2, placed in an RNA-stabilizing solution, or fixed. The ideal collection method to preserve the molecular composition of the tissue is sharp dissection without

heat, followed by snap freezing in LN_2 and long-term storage in −80 °C or per standard in LN_2 freezers.

2. *Eutopic endometrium*

   Eutopic endometrium can be collected using different methods including (1) an endometrial sampling device, (2) curettage with cervical dilation, if necessary, (3) hysteroscopic resection, (4) post-hysterectomy excision, and (5) brushing [25]. For detailed description of each collection method, see Fassbender et al. [26]. Menstrual cycle phase should be determined by an experienced pathologist, and the first day of the last menstrual period should be recorded on the biospecimen form.

3. *Myometrium*

   Myometrium is excised using cold scissors/scalpel or laser [25]. The recommended method of collection is through sharp dissection without use of heat to preserve the molecular composition of the tissue. Myometrium is then snap frozen in LN_2 placed in an RNA-stabilizing solution or fixed.

4. *Peritoneum*

   Peritoneum tissue can be collected using a brush (for collection of peritoneal mesothelial cells for cell culture) or surgical devices including electrosurgery, ultrasound energy, harmonic scalpel, laser, or cold scissors/scalpel. The recommended method to keep the molecular integrity of the sample is the cold sharp dissection without use of heat. The location of the sample collected should be recorded (see the EPHect Standard Surgical Form).

Sample Quality: Time and Temperature Between Collection and Storage

The time between surgical excision of the tissue and storage should be as short as possible [17]. In the WERF EPHect SOPs, it is recommended to limit this to 15 min to minimize enzymatic degradation. Although DNA is relatively stable [92], mRNA is particularly sensitive to degradation [120, 121], and phosphoproteins are also unstable [122]. The effect of tissue ischemia on RNA analysis is well documented for variety of human tissues [123–129]. Sheldon et al. demonstrated high-quality RNA for microarray analysis if the time between collection and preservation did not exceed 10 min [17]. Others showed that 15 min after collection 10–15% of all detectable genes and proteins and after 30 min 20% differed significantly from the baseline values [128]. An alternative to immediate snap freezing in LN_2 for RNA studies is to immerse the tissue sample into an appropriate RNA-stabilizing solution, which allows the sample to be temporarily kept at temperatures as high as 37 °C before long-term freezing. The time between tissue extraction and storage should be recorded.

Processing and Storage

The choice of processing via (1) immediate snap freezing in LN_2, (2) immersion in an RNAse inhibitor solution followed by freezing or paraffin embedding, (3) neutral buffered formalin fixation/universal molecular fixative and paraffin embedding (FFPE), or (4) in vitro culture depends on a number of factors, including the anticipated future use of samples, amount of tissue available, and budgetary constraints.

If interested in conducting analyses on a cellular subtype of ectopic or eutopic endometrium, which can be highly heterogeneous containing epithelial cells, stromal cells, fibrotic tissue, muscle tissue, and blood, microdissection can be performed on the tissues stored in RNA-stabilizing solution [130, 131], fresh frozen tissue, or FFPE tissue [132].

DNA is very stable and extractable from samples treated and stored within a range of methods including fresh frozen and fixation. However, it is documented that DNA recovered from long-term achieved FFPE samples is compromised in strand length [133, 134] due to the cross-linking properties of formalin, which could have consequences for technologic applications such as long-read next-generation DNA sequencing.

RNA is very sensitive to degradation, and multiple studies have investigated optimal processing and storage conditions [135–141]. The best storage methods are either immediate snap freezing in LN_2 or immediate immersion in RNA-stabilizing solution [135–137]. Tissue thickness is important for successful RNA stabilization for rapid and reliable diffusion of the stabilizing solution. In the EPHect SOPs, we recommend the sample is cut into slices not thicker than 0.5 cm.

In terms of long-term storage of tissue samples, in the WERF EPHect SOPs, we recommend to snap freeze the tissue as soon as possible after collection (within 15 min for RNA analysis) or otherwise immerse in RNA-stabilizing solution, followed by freezing in LN_2 or −80 °C freezers. Only if freezing for long-term storage is not an option, or large volumes of tissue allow for multiple storage methods, FFPE archiving is recommended.

Conclusions and Future Directions

The WERF EPHect initiative has provided consensus in endometriosis research on the surgical (SSF, MSF) and nonsurgical (EPQ-S, EPQ-M) data collection tools and SOPs (standard recommended and minimum required) for collection of fluid and tissue biospecimens along with a biospecimen form to collect additional data required for informative analysis of the samples. Adoption of these standardized tools and protocols by those conducting research in endometriosis will facilitate worldwide collaborations between centers and maximize validity of results.

All current WERF EPHect questionnaires and SOPs are freely available for investigators through endometriosisfoundation.org/ephect. The evidence base for all

of these instruments will be reviewed continuously based on feedback received from investigators adopting the WERF EPHect standards, along with regular systematic reviews of the literature and other publicly available evidence.

Centers utilizing the WERF EPHect tools can register on the WERF EPHect website (endometriosisfoundation.org/ephect) to enable cross center collaboration in endometriosis phenotype discovery. The development is currently underway of freely available software to facilitate center-restricted data entry and reduce costs and time expenditure to individual centers. It is requested that the centers publishing results using the EPHect instruments reference the sources and include version numbers of the instruments used in publications.

In conclusion, the WERF EPHect instruments were developed with input from leaders in endometriosis research and industry worldwide to facilitate large-scale, cross-center, longitudinal, epidemiologically robust, biomarker and treatment target discovery research in endometriosis. Integration of standardized phenotypic data collection instruments and adoption of the biological sample SOPs by research centers will enable large multicenter, geographically diverse studies with high reliability and validity to aid in shedding new light on mechanisms underlying this heterogeneous, enigmatic disease.

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