Thomas D'Hooghe *Editor*

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State of the Art

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Biomarkers and Endometriosis: The Need to Move Beyond Innovation to Validation

Preface

This book presents a state-of-the-art description on how much progress has been made in the development of a noninvasive test for the diagnosis of endometriosis.

Such a test is much needed, especially for the early detection of endometriosis of symptomatic women with pelvic pain and/or subfertility without evidence of endometriotic cysts, nodules, or adhesions on gynecological ultrasound. This would include nearly all cases with only peritoneal endometriosis, some cases of moderate–severe endometriosis without a clearly visible ovarian endometrioma, and cases with pelvic adhesions and/or other pelvic pathology undetectable by gynecological ultrasound. Ideally, this test could also substitute gynecological ultrasound and allow the diagnosis of endometriosis in areas where ultrasound is not available or reliable. The main aim of such a test would be not to miss any woman who might benefit from endometriosis surgery to improve pelvic pain and/or subfertility. In that context, a test is needed with high sensitivity (ideally more than 80%) and acceptable specificity, fully accepting the risk that a laparoscopy will be negative in some women [1]. This risk can be balanced by the advantage that a negative laparoscopy can assure women with pelvic pain and/or infertility that their pelvic anatomy is normal and allow other diagnostic/therapeutic approaches.

In this book, we start to describe the infrastructure/capability required for biomarker discovery and validation and then learn from experiences on biomarker discovery in pathologies related to endometriosis like cancer (endometriosis is a benign metastatic disease) and inflammatory diseases of the bowel, liver, brain, and cartilage (endometriosis is a chronic inflammatory disease). After highlighting the importance of patient centeredness in endometriosis care, we focus on epidemiology, risk factors, and genetic markers for endometriosis and provide insight into how OMICS and, more specifically, proteomics and transcriptomics have contributed to progress in this field. Extensive attention is given to the diagnostic performance of non- or semi-invasive tests for endometriosis based on (panels of) biomarkers in peripheral blood, peritoneal fluid, and eutopic endometrium. Recommendations of the World Endometriosis Research Foundation are summarized to illustrate the importance of internationally accepted standard operating

procedures for the collection, treatment, storage, and analysis of tissue samples and for detailed clinical phenotyping of these samples.

Two priorities emerge for the future on biomarker discovery in endometriosis.

Firstly, we need to move from innovation to validation. Indeed, in order to make progress for the benefit of patients, the most urgent task ahead is now to validate the diagnostic accuracy of any promising test prospectively in an independent symptomatic patient population with subfertility and/or pain without clear ultrasound evidence of endometriosis. This population should have a clinical indication for surgery (gold standard for diagnosis of endometriosis) and be divided into cases with laparoscopically and histologically confirmed endometriosis and controls with laparoscopically confirmed absence of endometriosis [2].

Secondly, more collaboration is needed between academic groups and industry to move from discovery via validation to clinical availability of biomarkers for endometriosis. Collaborative research efforts co-led by academia and industry, early on during clinical development of biomarkers, may accelerate validation of interesting biomarkers which is not sufficiently explored today. Most national/international research foundations focus so much on innovation and discovery and do not make funds available for validation. Both industry and academia should make an effort to prioritize resources and develop such partnerships for biomarker validation and development, building on their joint scientific and commercial excellence. In the end, patients will benefit from such collaborations, as long as they are scientifically sound and transparent [3].

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Building Translational Research Infrastructure and Access to Expertise for Biomarker Discovery in Cancer

Jacqueline A. Hall

Abstract The way that biomarker research is being conducted in oncology is changing. We are moving away from institutional and expertise specific silos to multicentre studies, networks of institutions and integrated multidisciplinary workflows. These changes are influencing the way that researchers operate and present a host of new challenges, both scientific and operational. Investing in translational research infrastructure represents both an investment in a technical platform and access to expertise to promote high-quality, streamlined procedures under appropriate governance. Key elements that should be addressed to facilitate the translational of biomarkers to clinical practice include sample collection, laboratory analysis, molecular and clinical data collection analysis and interpretation. Building these elements to create a supportive research environment is therefore becoming increasingly important.

Keywords Translational research • Infrastructure • Expertise • Biomarkers • Biobanking • Molecular analysis • Bioinformatics • Quality • Network

Introduction

Personalised Medicine and the Use of Biomarkers

There is an increasing drive towards understanding the biology of disease and drug response at a molecular level to be able to tailor patient treatment and management according to their individual molecular profile [1]. In addition, with the rapidly advancing capacity to quickly generate large volumes of molecular data at lower cost, there has been a tendency in biomarker discovery to use a battery of molecular tests to generate large volumes of molecular data. With this, there has been a lack of appreciation of the need for good biomarker study design and how to

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interpret the volumes of data generated, leading to contradictory results and experiments that have failed to validate. It is even possible that a large number of biomarker studies have never been attempted simply due to the lack of access to appropriate biosamples with the correct annotated data or restrictions due to ethical and regulatory issues that had not been covered at the time of collection [2]. There is now an increasing awareness of the need for good study design for biomarker discovery and also for implementing other required elements such as ethical, legal and logistical aspects that are necessary to support access to biosamples and associated data, thereby allowing the research to take place [3, 4]. Additionally, biomarker identification and utilisation are now becoming an integral part of drug development. For example, early identification and integration of biomarkers into clinical trials is now becoming widely accepted to enabling targeting the clinical trial design, allowing enrolment of fewer patients and increasing the likelihood of identifying positive responses to the drug [5]. Some recent examples of trials heavily based on molecular biomarker assessments include the BATTLE trial and I-SPY2 trial [6, 7].

The Need for Translational Research Infrastructure

The challenges associated with biomarker discovery and validation are now becoming well recognised. One of the major stumbling blocks has been the lack of successful validation of biomarkers. For a biomarker to progress to a clinically approved test that is implemented in daily clinical practice, a potential biomarker should be confirmed and validated using a sufficiently large number of cases to demonstrate statistical significance and should be reproducible, specific and sensitive. Hampering these efforts has been the lack of supporting infrastructure and limited or uncoordinated access to expertise to facilitate the biomarker discovery and validation process [8]. This has led to issues such as limited access to sufficient numbers of patients and sufficient quality of the right type of samples and associated data, inadequate controls, lack of access to appropriate statistical expertise and study design or address issues of limited clinical value. In order to support and enable the discovery of biomarkers, developing the appropriate infrastructure and providing access to expertise are required to facilitate and enhance research efforts. This represents not only technical aspects and tools but also dedicated resources and access to expertise in order to promote best practice standards and provide logistical and operational support and streamlined procedures under an appropriate governance framework. A supportive research environment effectively promotes integration of multiple disciplines including biobanking, clinical phenotyping, laboratory analysis, molecular data, informatics, statistical analysis and dissemination of results, all of which are required for successful biomarker studies (Fig. 1).

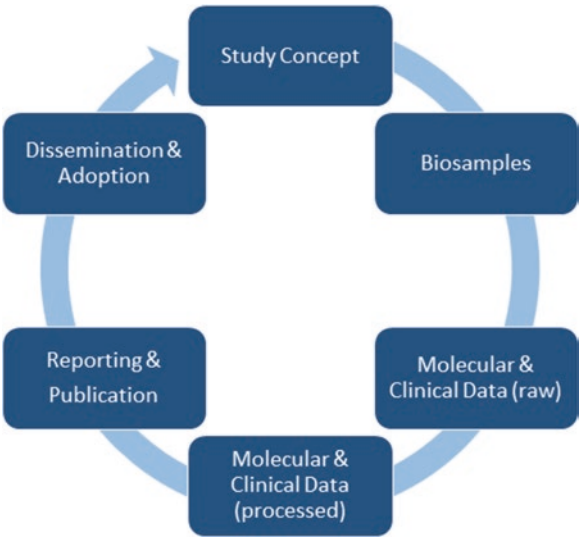

Fig. 1 Key elements of translational research infrastructure that support biomarker discovery and validation effort

Key Elements of Translational Research Infrastructure and Accessing Expertise

Clinical Use

Biomarkers are indicators of a physiological or pathological state of the body. They may be broad ranging, spanning from physiological measures like blood pressure through to molecular and chemical laboratory tests of blood, urine, saliva or other tissues. In all cases, by definition, biomarkers must be objective, measurable characteristics of biological processes and, importantly, can be put to use in multiple ways.

Biomarkers can be used in a number of ways, particularly to tailor patient treatment options (Box 1) as well as significantly contribute to our understanding of disease, in the development of new drugs and treatment regimens [9]. Given the variety of roles a biomarker may take, it is important to consider in each case if the assay methodology is fit for the intended purpose and to clearly define the clinical question and clinical utility of any tool that will be developed. Guidance for the design and reporting of biomarker studies in oncology has been developed, the principles of which could be usefully applied in other areas of biomarker research [10].

Box 1: Examples of Clinical Applications and Uses of Biomarkers

- Early detection (screening)
- Diagnosis and sub-classification of disease (classification)
- Prediction of the likely course of disease or outcome (prognosis)
- Prediction of toxicity to treatments or therapeutics (patient stratification)
- Monitoring of disease progression (monitoring)
- Prediction of therapeutic response (patient stratification)
- Understand drug metabolism and mechanism of drug action (pharmacokinetics and pharmacodynamics)

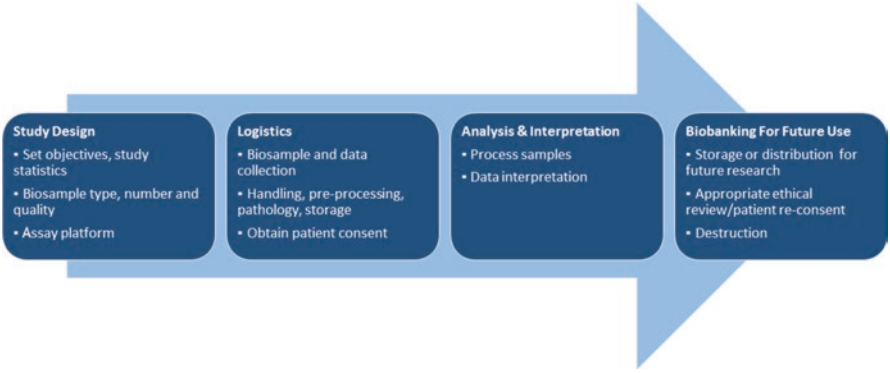

Fig. 2 The life cycle of a biosample involves many steps, a complex chain of participants and multiple stakeholders

Management of the Biosample Life Cycle

Performing any type of molecular profiling requires access to patient biosamples. However, establishing appropriate biosample collections is a complex task involving many different partners and steps (Fig. 2).

A number of biosample resources and guidelines have recently developed, for example, the ISBER best practices for biorepositories [11] and the P3G biobank resources including the comparison chart of guidelines [12]. These tools serve to create a thorough, accurate and standardised collection of information regarding the nature and manner of handling of biosamples, as well as providing information on the collection of the tissue itself and remaining materials available with appropriate consent for future research. At a minimum, a clear record of tracking the biosample from patient to fixation or freezer must be maintained. Biosample tracking and capture of biosample data through an appropriate biosample information management tool become paramount, not only to ensure an appropriate chain of custody but also

for capturing key information on sample handling that may influence the final analysis which cannot be standardised via standard operating procedures (SOPs), e.g. some pre-analytical procedures or deviations from collection protocols like accidental thawing. Recently standardised coding terminologies and tools have been developed to help researchers with cataloguing biosamples, for example, the use of biosample reporting systems [13]. Critically important for biosample collections is the link to the clinical information and follow-up data. This linkage can often be missing or incomplete in more mature biosample collections that are being used retrospectively for discovery research projects.

Quality of Biosamples and the Associated Data

Collecting and accessing sufficient numbers of biosamples has been a challenge to many involved in biomarker research, but what is often less well appreciated is the need to ensure that biosamples have been collected to an adequate standard of quality. Without appropriate collection protocols or documentation as to how the samples were collected, a biosample may be unusable for the intended purpose, and collection protocols should be grounded in scientific evidence wherever possible. The type, amount and quality of the biosample required can vary greatly depending on the type of test to be performed [14].

Broadly speaking, two main approaches for the collection of biosamples for biomarker research can be applied: retrospective biosample analysis and prospective biosample collection. Retrospective biosample analysis involves the use of pre-existing collections and can be useful for quickly investigating open questions and may also have the advantage of longer follow-up information that has been collected over a period of time but which is now available for immediate use. Retrospective biosample collections can be a valuable tool for identifying distinct molecular subgroups of patients that show different profiles and clinical phenotypes or which benefit from different therapies. One well-known example from oncology is the analysis of KRAS mutation status in colorectal cancer which was shown to predict monoclonal antibody therapy response by retrospective analysis of previously collected biosamples. Also the methodology used to detect KRAS mutations is one that can be used on standardly collected clinical materials, such as the use of formalin-fixed paraffin-embedded (FFPE) material without the need for specific tissue collection protocols being implemented [15]. Prospective collection involves the collection of new sets of biosamples specifically selected to answer a particular question. This process may take longer and use more resources than retrospective analysis of biosamples; however, prospective collection has the added advantages of better control over the parameters of biosamples collection (and these may be tailored for the specific test method planned for use) and is often used in the context of clinical trials [16]. As this type of collection is more focused, it can provide a greater degree of confidence in that the right type and sample specifications will be implemented for the biomarker study in question. Prospective, protocol-driven biosample

collection will also be absolutely necessary when moving forward for the purposes of validation, where studies are designed and driven by hypotheses. In this situation access to the biosample may be absolutely required for patient enrolment, and so this leads to more stringent requirements regarding the quality of collected biosamples than is sometimes applied in the case of discovery research. For example, to run a gene expression profiling array, carefully collected frozen tissue, frozen within 30 min, is usually required [17] but which then requires specifically implemented tissue collection protocols.

This was the case with the samples collected in the MINDACT (Microarray In Node-negative and 1–3 positive lymph node Disease may Avoid ChemoTherapy) trial, which is a study that exemplifies the inclusion of genomic risk profiling in clinical research and which required careful implementation of biosample collection to allow genomic profiling as part of the study goal. The aim of the trial is to provide further evidence that early breast cancer patients with a low recurrence risk genomic profile by MammaPrint® (a 70-gene expression signature) may not need chemotherapy. Due to the study design, it was mandatory for all patients involved to have fresh frozen tissue samples collected and tested with the genomic signature. Since access to high-quality frozen tissue biosamples was absolutely essential for patients to participate in this trial, optimised tissue collection protocols needed to be designed and the challenging logistics well managed [18]. The challenge was met through the development of strong interactions between the different departments involved in the conduct of the trial, e.g. medical oncology, surgery and pathology, as well as a trained research nurse/fellow. Over 6500 patients were enrolled in this trial, and over 6000 frozen tissues were collected and analysed. Furthermore, over 90% of enrolled patients gave their consent to use biobanked tissues for further research.

In any case, care must still be taken that the chain of events is appropriately managed and information recorded at appropriate time points and that this meets the required needs of the study. Throughout the process of collecting, processing and storing biosamples, materials are subject to a number of factors that can significantly alter their molecular composition. These factors range from physiological, e.g. effect of lifestyle, age, gender and endogenous variables, such as exposure to drugs that influence biosample quality prior to removal from the research participant, to factors that arise at a later point in the process such as handling, processing and transport from one location to another (e.g. from the hospital to the laboratory conducting the analysis). Poor or inappropriate biosample quality can be directly responsible for incorrect determination of molecular and/or physical characteristics during subsequent biomarker analysis. Therefore appropriate biosample collection with evidence-based standard operating procedures that are fit for purpose needs to be established [19]. This may require some background work in biopreservation and storage methods and how these affect the molecular readout from the sample, taking into account the type of laboratory technique that will be applied, and becomes particularly important in multi-institution collections where variations in practice may occur.

Selection of Collected Biosamples

Access to the right type of biosamples is also fundamental to applying personalised treatment in the clinic. This includes using clinically relevant biosamples for biomarker discovery purposes such that any subsequent tests developed in this context can be applied in a clinical setting without the need to migrate to a new type of biosamples or new test method. With this in mind, it is often best to consider collecting a range of different types of biosamples such as primary tissue from the site of the disease, whole blood, serum, plasma or other body fluids like saliva and urine or peritoneal fluid. Some of these sample types require less invasive methods to collect that impose less of a burden on patients, making them easier to implement in the clinic and are also less expensive. An additional advantage to less invasive biosample collection is that duplicate or triplicate samples may be taken at the same time point enabling a range of different research studies or different types of test to be performed. Also samples may be taken repeatedly over time allowing longitudinal monitoring of biomarkers through the course of disease and monitoring the effect of treatments.

In addition to well-characterised gold standard biosamples taken from the primary site of disease, such as histologically confirmed primary tumour tissue, having surrogate tissue sample collections like blood, saliva or urine taken from the same patient can be very valuable in understanding the concordance between surrogate tissues and the primary tissue. For this reason much attention has recently been focused on cell-free circulating tumour DNA (ctDNA) or circulating tumour cells as a way of obtaining a 'liquid biopsy' [20, 21]. Matched adjacent normal tissue can also provide useful insights for comparison to the tumour itself. Having access to a broader range of biosample types can be particularly important depending on the intended clinical use of the biomarker and the question being addressed with the biomarker study. For example, addressing the question of sub-classification of the disease into different categories will require a full spectrum of patients to be represented in the cohort so that the different categories can be identified. Additionally, biosamples from unaffected individuals can act as control samples. During ongoing biobanking efforts, particularly in cohort-driven study protocols, care should be taken to avoid the introduction of bias due to the ease or difficulty of collection of particular sample types or ease of access to particular types of patients. Thus links to well-characterised clinical phenotype information also become critical.

Accessibility of Biosamples

As diseases are diagnosed earlier and tissue sampling methods improve, the quantity of tissue material available for research purposes is declining. In cancer treatment, although diagnostic biosamples are routinely collected in hospitals as part of the standard care of the patient, these are not always available for research as the primary healthcare needs of the patient take priority for use of the specimen. If access to

diagnostic specimens is requested for clinical research, this can necessitate sending diagnostic material outside the clinic, at least temporarily. Alternatively, if sufficient material is available, the tissue sample may be divided; however, in some situations, the pathologist may deem that division of the material is simply not possible due to the small quantity of starting material or low tumour cell content. In contrast, for certain cancer types such as for some gastrointestinal cancers, large volumes of material may be removed from cancer patients as part of the standard treatment, and portions of this residual material may be kept for research. In addition, the general fitness of the patient may influence the ability to collect biosamples depending on how invasive the procedure is and where the tissue needs to be taken from.

Other factors such as ethical and legal requirements can also pose constraints on the use and access of biosamples for research, and this must be handled in an appropriate manner. Notably, the entire biobanking process requires appropriate oversight and management in accordance with the prevailing regulations and applicable ethical principles, such as appropriate consent and/or approval by an appropriate body, e.g. research ethics committee, as applicable. Within an institution, having dedicated processes and reaching agreement between the various stakeholders such as surgery and pathology departments that are involved in the handing and decision-making process around the tissue samples can better ensure that the best use of the available biosamples can be made in line with good clinical practice requirements. To facilitate this for multisite collections, master contracts and agreements with participating sites can be extremely useful for the articulation of governance and regulatory regimes. Importantly, having appropriately trained staff, such as a research nurse available on site, can facilitate the consent process and ensure a robust chain of custody for the sample.

Future Use of Biosamples

Collection and storage of biosamples for future access provides an important resource for biomarker discovery and validation [22]. When a biosample is collected, part of the tissue may already be allocated to a specific immediate use in which case appropriate standards for the collection of the biosamples can be defined according to the end use; however, there may be residual material left over that could be stored for future research purposes that are not yet defined. In order for this residual material to be useful, care should be taken to document the process in order to avoid any use of the biosample outside the scope of informed consent and also to be able to subsequently link clinical or other annotations to the right tissue. For the purpose of biobanking biosamples for the future as yet unknown research, the applying biosample collection SOPs become challenging. Under these circumstances it is often best to revert to standard best practice guidelines for biosample and to collect biosamples in a manner that is as close as possible to those observed in current good clinical practice.

Clinical Phenotyping

As well as collecting and accessing appropriate biosample collections, it is critically important that biosamples are well annotated with clinical phenotype data and follow-up information. This is especially important when the phenome of the disease needs to be defined and better characterised. Detailed clinical information such as data that may be available in patient records or electronic files should be collected including information such as age, diagnostic data and detailed surgery report with scoring and staging. Additional lifestyle and epidemiology data or quality of life data can also be collected by way of standard questionnaires. Standard coding systems for capturing clinical information such as the Systematised Nomenclature of Medicine Clinical Terms (SNOMED CT) [23] are available.

Biomarker Analysis

A Variety of High-Throughput Platforms for Molecular Analysis

In addition to traditional laboratory techniques such as immunohistochemistry (IHC) and polymerase chain reaction (PCR), recent technological advances in omics and multiplex technology have led to the possibility of simultaneous analysis of a large number of biomarkers in a single experiment using less and less biosample material. A good example being next-generation sequencing that has the capacity to rapidly generate a vast amount of data on the molecular profile of the tumour or obtain the entire genome sequence of the individual [24] in a short space of time and now also at reasonable prices. This has led to many advantages; large volumes of data have been generated and significant results have been derived from these; however, a limiting factor in this process has been the lack of characterisation and validation of such technologies in practice and moving exploratory newly discovered biomarkers from omics technology into robust and validated markers ready for use in the clinic [25]. Therefore further steps need to be taken for making the transition through to robust and clinically useful biomarkers.

Assay Robustness, Reproducibility and Analytical Validation

Assay robustness refers to the ability of a test to provide consistent results using independent samples and provide confidence that minor perturbations in conditions do not greatly affect the results of the test. Robustness is an issue that has been raised in the bioinformatics community around methodology for biomarker signature development from high-throughput data. Due to redundancy and rich, complex datasets, different subsamplings of data may lead to very different results for selecting biomarkers. Methods have recently been proposed to help

address the issue of stably selecting features from large volumes of data [26]. Reproducibility of an assay refers to a biomarker test that can reliably give consistent results and the same classification of cases by different people, for example, in different laboratories.

Analytical validation refers to the ability of the test to accurately and reliably measure the analyte or genotype of interest, for example, a panel of biomarkers designed to evaluate a set of mutations in the *BRCA1* and *BRCA2* genes to test for hereditary mutations. Analytical validation is essential for ensuring the consistency of the test's ability to measure the specific biomarker of interest. Assays should be suitably analytically validated for their intended purpose [8], i.e. the performance of the assay should be demonstrated and the assay is finalised and 'locked down' prior to use [5]. In Europe, recent guidelines have been issued from the European Medicines Agency (EMA) [27]. Additionally, part of ensuring assay robustness also includes maintaining appropriate documentation such as detailed SOPs or other related laboratory guidelines. This is a good practice even at the early discovery stage, ensuring a traceable link between the collected biosamples and the data generated which can be invaluable when the resultant data are being analysed and interpreted.

Clinical Validity and Clinical Utility

In addition to robustness and analytical validity, biomarkers must also demonstrate clinical validity and clinical utility in order to be translated into clinical practice. Clinical validity refers to the accuracy with which a test can predict the clinical phenotype of interest. In order to demonstrate this, epidemiological studies or clinical studies are required that can generate substantive evidence measuring the strength of association between the biomarker and a specific and well-defined phenotype which allows estimation of the sensitivity and specificity of the test. An example of a test where substantial evidence for clinical validity was achieved is the use of the Oncotype Dx 21 gene signature for providing clinically valuable information to predict effectiveness of chemotherapy in ER-positive breast cancer patients, in addition to standard measurements (e.g. tumour size, grade, lymph node status and single molecular markers such as oestrogen receptor, progesterone receptor and HER2) [28, 29].

Clinical utility refers to the ability of the test to change patient management and demonstrate that it will significantly improve health-related outcomes. This also often involves comparison of the test against current gold standard methodologies. Well-designed and conducted studies are needed to demonstrate this and should be done in a representative population. Systematic review of existing data and meta-analysis of randomised controlled trials showing consistency in results can also be used but may provide a lower level of evidence than prospectively designed studies. Documents detailing validation data and experimental results should also be available along with a background or rationale, data and supporting publications showing the clinical validity and clinical utility of the test for the role in the trial [5].

The concepts of analytical validity, clinical utility and clinical validity for different types of biomarkers have been well summarised by the Evaluation of Genomic Applications in Practice and Prevention (EGAPP) Working Group [30].

Finding solutions to support researchers with the challenges of validating biomarkers and assay methodology is challenging, and various approaches are in operation. For example, the Cancer Research UK has developed road maps outlining the expectations for cancer biomarker projects that are submitted for grant funding [31], and the NCI USA has developed guidance and a study checklist for those implementing biomarker tests in phase 2 and phase 3 trials [32]. Another approach is to provide supported access to appropriate analysis facilities composed of dedicated certified laboratories rather than providing grant funding for the work to be carried out locally, as is the approach of the Clinical Assay Development Program (CADP) of the NCI USA. This approach provides an environment for appropriate development of fit for purpose tests and dedicated project management support to assist with coordination of development projects.

Data Management

Clinical Data

The collection and management of data for clinical research is a mature field, and several current standards exist, for example, the Good Clinical Data Management Practices (GCDMP) guide developed by the Society for Clinical Data Management (SCDM) for data management in clinical trials [33]. Various procedures for data management in clinical trials include the design and annotation of case report forms (CRF) which collect the necessary core details about the participants in the study required to meet to research objectives, clinical database design, data-entry procedures, processes for data validation, discrepancy management, medical coding, processes of audit trail to track data origin and modifications, data extraction and database locking. During the conduct of clinical trials, these processes and data quality are regularly checked at intervals. Standard clinical information such as diagnostic information, treatments, dosage, clinical outcome, etc. are therefore often well managed within standard clinical databases. However, in other settings other than well-defined clinical trials, the challenges of collection of long-term clinical follow-up can be much greater. Using the basis of these well-established methodologies from the field of clinical trials for developing and using clinical data resources for biomarker research is a distinct advantage and provides assurance of the robustness of data collected.

In recent years, electronic data capture (EDC) has grown considerably and offers advantages for uniformly capturing data from multicentre studies, automated de-identification of data, study management and electronic data checks. EDC is also facilitating the collection of data that has previously not been easily accessible, for example, through the use of applications or programmes implemented on touch-screen

devices, like tablets or mobile phones, or interactive voice response systems on telephones that can even be used to collect patient-reported data. Various different commercial and open-source tools that offer similar functionality are available due to the high demand and the need to meet regulatory criteria. Examples include Oracle Clinical, Clintrial, RAVE and eClinical Suite (commercial) and OpenClinica, openCDMS, TrialDB and PhOSCo (open source and free of charge). In addition, data management tools now need to be extended to cover aspects including provisions for workflows for biosamples, their associated data and molecular data.

Biosample Metadata

Consistently recording and tracing biosamples through multistage analytical processes is important for maintaining the biosample audit trial and to enable future use of the biobanking resource. Data about the biosamples (biosample metadata), including both logistical information such as location, shipment batch number, date and time of delivery and quality information such as lag time from collection until freezing, preparation method, percentage tumour cells for tumour samples and tube type for blood collection, are needed. A minimum dataset for sharing biobank samples, information and data has been developed that can assist with harmonisation and interoperability of sample information facilitating exchange between centres [34].

Several organisations have also now developed software tools specifically for managing biosamples data. Some examples include biosamples databases from the National Center for Biotechnology Information (NCBI) [35], the European Bioinformatics Institute (EBI) [36] and the International Association for Research in Cancer (IARC) [37].

Molecular Data

Given the large volumes and variety of molecular data that is now available from high-throughput techniques [38], the management of molecular, clinical and biosample metadata is a new emerging challenge. This task can be approached in different ways. One approach is to centralise data and have central coordinated management; however, this requires securing adequate storage space, tools and expertise to enable management or large volumes and diverse types of biological data and also management custodianship of data that may have been generated in different locations or institutions. The resources required for centralisation can be particularly significant in the case of new technologies such as next-generation sequencing, not just for physical storage but also in personnel time for curation. A second approach is to use a federated model where data is not physically centralised but instead storage locations are linked. This approach can more easily take advantage of existing public data repositories such as the European Genome-phenome Archive (EGA) [39]. Notably, greater accessibility, awareness and harmonisation

between available bioinformatics resources have been identified as a key area for further development within Europe, as exemplified by the opening of a new pan-European bioinformatics infrastructure for biological data called ELIXIR [40].

Implementing common data standards has been proposed as a way to save time and resources in data collection as it facilitates interoperability of systems and lends itself to automation. This can increase the quality of data and the ability to reuse data for secondary research projects by facilitating data exchange. Several international initiatives for inter-institutional data harmonisation are currently underway, such as open data standards developed by Clinical Data Interchange Standards Consortium (CDISC) [41] and the Critical Path Institute (C-Path) which orchestrates the sharing of data, expertise and knowledge among industry, regulatory authorities, government, patient advocacy groups and academia in the precompetitive space to generate the evidence needed to improve the drug development pathway [42]. However, for successful implementation of such frameworks, the community must be engaged and come to an agreement on which standards to adopt since parsing and organising the data according to set standards takes an initial investment of time and resources.

Interoperable Databases and Information Technology

Interoperable databases and supporting integrated computer solutions are an often underestimated but foundational building block for supporting biomarker translational research. With the rapidly advancing capacity to quickly generate large volumes of molecular data at lower costs, information technology (IT) platforms for organising, storing and linking data in a robust and secure way compliant with appropriate ethical and confidentiality rules become critical. One of the major challenges of building IT solutions is the diversity of the data and formats to be collated, particularly if the data are sourced from different institutions or partners. Data can range from large volumes of molecular results to clinical outcome or biosample data or even administrative and cost information. Increasingly, projects require the ability to link between datasets or operations to allow cross database searching, for example, combining molecular and clinical information to identify patient groups where biosamples suitable for future research studies are available in the biobank.

A variety of data types and formats need to be integrated into an interoperable system that allows flexible queries. As part of developing such IT platforms, formalised data structures, ontologies and common data elements/minimum datasets become critical for efficient linkage, retrieval, interpretation and exchange of data.

Capturing data in a modular way allows flexibility in search functionality, and data retrieval in a format can meet the needs of various user groups. Data may need to be downloaded, shared or placed in the public domain, as appropriate. Data distribution must be managed according to data access rules, contractual restrictions, the scope of patient consent, data access rights of users and institutional and custodianship restrictions; therefore, having tools that can help manage this process is a

great advantage. For example, new tools such as electronic watermarks can be used to facilitate traceability of the origin of data and how it is used.

Whether the selected solution is a one-stop-shop platform or involves combining a series of different software, given the diversity of queries, interoperability between systems is key. Functions for workflow management and repeatable automated report generation that build and record the sequence of steps become important for traceability and integration.

Finally, after collection, data must be analysed and interpreted in order to gain knowledge; therefore, the interface with analysis and visualisation tools becomes important [43]. Common private or public cloud-based workspace tools can also facilitate data analysis between investigators who are physically situated in different locations but who may be working on the same data and software solutions that assist in managing, visualising and analysis of diverse translational research data. These platforms can also provide for the sharing source code or analysis algorithms between investigators and research centres that serve to map the raw data to the results via audit trails. These tools may be either integrated into the IT platform or may be articulated as separate stand-alone software. Since numerous analysis software (both open source and propriety) are available to researchers, data should be accessible for downstream use in a way that allows researchers the freedom to select the analysis tool of choice. Critically, whatever IT solution is selected, it must have a user-friendly interface in order for it to be adopted.

Data Analysis

New challenges in the field of data analysis are emerging now that the field is moving towards a holistic approach to medicine and biology. Through the integration of multiple data types, we may allow more precise, robust and meaningful identification of new cancer biomarkers [44].

Biostatistics

In the past, poor biomarker study design has led to a large number of spurious conclusions, and contradictory results are frequently found in the published literature. Methodological issues stemming from small sample sizes and multiple hypothesis testing have resulted in a loss of power and inflated estimates of statistical significance of putative biomarkers [45].

In addition, the effect of technical variations and pre-analytical variations on final test results and how they can lead to serious biases and obscure the effects and variation of interest is becoming increasingly appreciated [46]. These issues become particularly prominent for multiplex biomarkers, such as gene signatures. It is important that the biomarker research is robustly designed and that the statistical analysis methods are clarified and described in sufficient detail to allow

up-front consideration of the power and sample size, and so early consultation with a statistician is advisable, and the type of study design selected can affect the level of evidence generated from the study [4]. Even if the biomarker study is exploratory in nature, some rationale for sample size should be given of why that number of specimens should be collected or better still the biomarker study should be designed to detect a specified effect size, e.g. a specified hazard ratio. Initial sample size should be estimated wherever possible. A key parameter impacting sample size estimation is the frequency of occurrence of the biomarker (e.g. an infrequent biomarker requires a large sample size to achieve the same power), which can greatly influence the feasibility of accessing the required number of specimens to perform the work. Care must also be taken in considering the type of data produced by the assay, how the final scoring of the results will be categorised and what cut-points will be used to determine these categories. This is an important issue if the biomarker is to be used to assist with treatment decisions and subsequently impacts on the statistical design and power calculations.

Variability in biosample collection, storage, processing and analysis can have a major impact on the quality of the data obtained and if not well managed could lead to artefacts resulting in misinterpretation of test results. Therefore, even when working in a biomarker discovery setting, it is important to consider that assays are well characterised and validated and that parameters around the biosample collection and analysis are not associated with artefacts or lead to confounding in the data produced, for example, effects of the type of tube used for serum collection or deviations from sample collection protocols such as sample defrosting that may lead to effects in the molecular data produced.

The type of assay platform selected may differ between a discovery and validation setting, for example, in a biomarker discovery project, a method that produces a large amount of data in parallel may be selected such as genomic profiling or genome sequencing methods. Less robust assays may be more sensitive to subtle variations in the biosample collection and preservation methods making the need for well-documented, standardised collection protocols important.

Subsequently for validation objectives, once a smaller set of biomarkers have been identified, robust clinically applicable assays that measure only a few biomarkers may be used. These qualification studies may also often performed in a surrogate tissue other than that of the original disease site such as serum or blood. It is also worth considering the expected failure rate and failure distribution of the assay as this may be important early indication for assessing the feasibility of the implementation of the biomarker. Assay failure rate can impact on the number of patients that need to be measured as part of the study but also if there may be bias of particular patient groups that are not assessable for the status of the biomarker.

Given these potential pitfalls, it is important to have early statistical input into the design of biomarker studies. Ideally, and in order to reach the highest level of evidence, analysis plans should be prespecified. This can also be done when the biosample collection is not collected in a prospective manner (known as a prospective-retrospective design) [4].

Bioinformatics and System Biology

It is now recognised that both biostatistical expertise and bioinformatics expertise are complementary and necessary part of biomarker research particularly in the case of complex multigene classifiers. A good example of the needed intersection of both areas of expertise is the use of clinic-genomic predictors in clinical trials to determine patient treatment. Recently the NCI USA proposed criterion for this scenario that drives home the importance of rigour in all aspects of the analysis and verifying data accuracy, completeness, screening for artefacts in the data, locking down the algorithm, summarising the distribution of predictions and method validation including comparison against public sources of data [47]. Verification would also include monitoring of data management and checking raw data formats. Commented source code is recommended to enable a skilled reader to follow the algorithm and written descriptions to document data transformation steps being performed [3]. Literate programming tools are available, such as Sweave, SASweave and odfWeave that can assist in documentation of code [48].

Verifying Quality and Expertise, Quality Assurance and Quality Control

Quality Assurance of Laboratory Testing

Quality assurance and quality control are an often under-appreciated but important part of biomarker research. Increasingly certification and accreditation of facilities is becoming important to harmonise activities when work is conducted in a decentralised manner across multiple institutes as it provides some confidence that the partners are working to similar standards.

One route is through the accreditation of laboratories to internationally recognised standards such as those established by the International Organization for Standardization (ISO) [49]; however, in addition to this, internal and external quality control is also important. Internal controls such as the use of control biosamples and comparison of their values against control limits should be an integral part of the assay testing procedure that is implemented locally. In external quality control programmes, the data of control biosamples are submitted to an external organisation for evaluation. This type of programme serves to monitor long-term assay performance within a laboratory and allows investigators to interact freely within the network to discuss technical issues, thus helping to improve the overall quality.

Quality Assurance in Biobanking

Although consensus on international technical standards and accreditation has lagged behind in the field of biobanking, new approaches for certifying or accrediting biobanks and developing tools are ongoing, for example, the ISBER Self-Assessment

Tool (SAT) for repositories which is available as an online self-assessment tool [50]. In addition, other standards such as a data standard for sourcing biological samples in the setting of biobank networks have been released [51]. These examples cover the general principles of quality management systems such as equipment qualification, validation of methods, resources, facilities, staff training, traceability, documentation, reference materials and participation in proficiency testing as well as enabling communication about specific samples and aggregated data which form important aspects of high-quality biobanking [52, 53].

Logistics and Operational Support

Conducting biomarker research requires coordination of multidisciplinary teams, and in the setting of international or multisite research, additional challenges may arise. Therefore, dedicated operational project management support is highly beneficial to support biomarker research.

A recent example of addressing feasibility and operational challenges associated with implementing biomarkers in clinical practice is the UK Stratified Medicines Programme. This programme has focused on establishing the collection of cancer biosamples according to given standards, performing centralised testing and securing turn-around times in a clinically relevant timeframe coupled with collection of a minimum pathology dataset and centralised storage of data and has tackled key hurdles with practical implementation of profiling such as timely and practical patient consent, access to samples and adequate preparation (including national pathology guidelines), informatics and interoperability of IT systems and communicating test results. Over a period of 2 years up until July 2013, over 9000 people had their tumours profiled over a range of cancers including melanoma and breast, bowel, lung, prostate and ovarian cancer. This has resulted in 8000 patient records that are securely stored in a research database, with planned access for researchers [54].

Operational tools and checklists for project management of biomarker studies may be useful for managing complex projects such as the integration of biomarkers in oncology clinical trials [55].

Governance

An often overlooked aspect of biomarker research that is key to the success of any biomarker project is the coordination of the multiple experts and disciplines involved whilst accounting for local regulatory and conforming to appropriate applicable ethical principles. Key issues that arise that need to be managed appropriately include obtaining and tracking the appropriate informed consent accompanying biosamples and data, confidentiality and privacy of the participants' identity, managing the return of results to participants (and deciding if that is appropriate), specific aspects relating to research involving biosamples taken from children, the use of

biosamples and data by commercial entities, the involvement and responsibilities of ethics boards and custodianship of biosamples. Therefore, the research governance framework establishing clear guidance as to the use of biosamples in research covering these issues in addition to the standard safety concerns of handling human materials is an important element in supporting translational research infrastructure and is essential for maintaining public trust in researcher's activities.

Governance coordination is particularly important in the case of inter-institutional cooperation when multiple entities and jurisdictions are involved, ensuring that work is in line with prevailing legislation and local regulatory needs. Oversight of the process by way of policies or guidelines for researchers can be useful for defining the roles and responsibilities of the various stakeholders involved helping to create transparency by establishing mutually understood ground rules in collaborative groups. Elements such as quality control measures, risk assessments and monitoring plans can also be built into this managerial oversight to help manage any potential roadblocks. The major challenge in establishing good governance is to maintain streamlined processes that are simple and practical for researchers to comply with, or else innovation and cooperation between researchers may be stifled.

Ethical Principles for the Use of Biosamples in Research

Access to human biosamples for use in research is essential for biomarker discovery and validation. The core principle that freely given informed consent obtained from each patient for the use of biosamples in research is widely recognised in the global research community. Therefore, there will always be cases in which the patients not wish their biosamples to be used in research, and this must be managed appropriately to maintain public trust. There is, however, considerable variability in how this fundamental principle is understood and applied in different countries. For example, a DNA sample in one country may be available for use in any well-founded research study, whereas a similar DNA sample in another country may have tight restrictions on the scope of its use, and a specific consent relating to genetic research may be required before it can be processed. As part of the normal process for obtaining informed consent, patients may be asked to consent to a predefined use of the biosample in a specific research study, if this is already known, or they may be asked to consent to an open-ended objective where the final use of the biosample is not yet known. In the latter case, when a new biomarker research proposal is proposed, the research would then be reviewed by an appropriate ethics committee. It is not always possible to implement this latter type of open-ended consent, due to differences in perspective of different ethics committees in different jurisdictions. For example, some ethics committees simply may not approve of such open-ended questions being posed to the patient. Furthermore, in some countries, researchers may even be asked by ethics committees to recontact the donor (if still alive) to obtain a new consent for any new research proposal. Clearly these ethical and legal obligations can add to the complexity of performing biomarker research yet must be handled appropriately. This means that dedicated regulatory support and associated guiding governing principles need to be put in place to fulfil our responsibilities to

patients; therefore, access to dedicated staff to assist with these processes can greatly facilitate the process and remove some of the burden from individual researchers. A variety of governance models exist among different organisations; however, the principle of custodianship, the entity or person responsible for the caretaking of the biosample, remains a common theme [56]. In some models, the custodianship of the biosample is dictated by the physical location of the biosamples (e.g. confederation of cancer biobanks), or the custodian may be defined by a legal entity, e.g. institution or hospital (Medical Research Council, UK) or with individual responsible persons (GlaxoSmithKline), or may be defined as the body deciding on the use of the biosamples (EORTC). In any case, the relationship and responsibilities must be clarified as these may vary between organisations and should be agreed upon when collaborations are initiated.

Use and Reuse of Data in Biomarker Research

In addition to obtaining informed consent from patients for the use of biosamples, consent for the use and reuse of clinical and biological data in biomarker research must also be appropriately governed. In the context of an environment where collaborative projects are increasingly important and funding agencies and scientific journals are encouraging researchers to share data, researchers need to be aware of the conditions under which this is appropriate and may benefit from dedicated translational research infrastructure support in order to do so. Data exchange and storage processes must comply with the prevailing regulations on privacy, confidentiality and security since they may be regarded as personal and sensitive data. Data should not be identifiable to an individual, but should be coded or anonymised in an appropriate fashion. For the case of some of the newer technologies like next-generation sequencing, this has led to new discussions regarding the identifying nature of the data itself and how to manage this [57]. Medical data are considered sensitive information, and debates still continue regarding appropriate models for (re-)accessing medical data for research, for example, whether patients should be re-consented for secondary use of data in new research projects. Additional regulatory challenges may arise when exchanging data and biosamples outside Europe, especially to the USA, because of differences in data protection laws. Novel methods in computing and software solutions can help support this process, for example, the project DataShield exemplifies an approach that allows parallel access to and analysis of data that are physically distributed in various locations [58].

Other common practises for managing data access include controlled access policies where researchers must demonstrate their competence and need for access to the data or where data is released only to restricted sets of individuals such as the National Institute of Health's controlled access data contained in the database of Genotypes and Phenotypes (dbGaP) [59]. Also new models of consent such as the concept of dynamic consent are emerging which could fundamentally change the existing model of consent and information exchange. These trust-based models encourage more feedback of information to participants providing samples to foster transparency and thereby allowing broader consent models to be applied for data reuse without having to re-consent the patient each time [60].

Future Trends: New Ways of Working

Industry-Academia Partnerships

As we enter a new era of collaborative research and cost sharing, new models of partnership are surfacing. Within the commercial sector, there has been some movement to promote precompetitive collaborations between companies that offer sharing of selected data and joint troubleshooting of fundamental issues that offer no competitive advantage to any one firm, and so by solving the issues jointly, all parties benefit by reducing costs. Having dedicated translational research infrastructure and access to expertise promotes this process by supporting the adoption of best practices, improved clinical decision-making, less costly clinical studies, development and optimisation of information technology, improves quality and provides access to expertise and therefore generates value in this precompetitive arena that can lead to cost savings. The improvement of processes that this coordination provides creates value that can be of interest to a range of stakeholders and is not limited to commercial entities; it also benefits including patients, governments as well as academic researchers, and opportunities arise for collaborations and synergies as new stakeholders become involved.

Additionally, in recent years there has been increased interest in public-private partnerships for jointly tacking healthcare issues [61]. Some examples of public-private partnerships in the area of biomarker development include that of the Dutch Technology Foundation, which has awarded a grant of 4.3 M Euro to establish the Biomarker Development Center, a public-private partnership involving seven participating industrial partners contributing both in cash and in kind. The goal of this initiative is to accelerate the validation and development of previously identified biomarkers in Alzheimer's disease, COPD and type 2 diabetes [62]. A second example of a public-private collaboration in biomarker development is that of the Unbiased Biomarkers for the Prediction of Respiratory Disease Outcomes (UBIOPRED) consortium that aims to validate innovative testing methods to classify patients into different subtypes of severe asthma as part of the Innovative Medicine Initiative (IMI) [63].

Open Innovation Applied to Biomarker Signature Discovery

Other new concepts that have also impacted on biomarker research include the concept of crowdsourcing which has been used as a completely new approach to tackling the identification of novel biomarker signatures. Crowdsourcing is the process of soliciting services, ideas or content from a large group of people, such as an online community. An example of its application in the field of biomarkers is an innovative programme called the Sage Bionetworks/DREAM Breast Cancer Prognosis Challenge [64]. In this challenge, web access was granted to a breast

cancer dataset that could be used to develop algorithms and signatures to predict breast cancer clinical outcome. The algorithms were made available as executable source code. Participants were able to login and use the data to build various models using their own choice of computational methods, then the predictive value of the different models were compared using the same standard statistical approach and the results were posted on a public board. The best algorithm was selected by validating the models in an independent dataset to give a robust estimate of performance. The winning approach was then published in Science Translational Medicine. This example of a new way of approaching clinical challenges through a scientific common which could radically change the way science is conducted in the future.

Collaborative Platforms for Harmonisation and Exchange

In order to conduct high-quality biomarker research within a reasonable timeframe, today's biomarker researchers cannot work in isolation. Multicentre collaborative efforts are often needed to achieve access to sufficient data and access to biosamples in order to meet the study design and statistical power requirements. Therefore, networks and consortia of researchers are now being established where shared access to biosamples and data from multiple institutions takes place. New platforms are now emerging such as Seven Bridges Genomics, with technologies to facilitate collaborative and large-scale data analysis, visual interfaces to make data accessible and ensuring connectivity and reproducible research via the use of Common Workflow Language and recognised standards [65]. Adoption and coordination of technical standards is essential for moving forwards in this domain and enabling meaningful exchange of data and accelerating scientific advances, particularly in supporting research in rarer diseases or large-scale population-based research.

Recognising the Benefits of Sharing

As well as harmonisation of technical criteria to allow meaningful interchange of data and biosamples, social factors may also influence the willingness of researcher to share biosamples and data, and these aspects are as equally important to manage as the technical factors. The custodians of data and biosamples need to be convinced and motivated by the synergies that can arise from sharing resources within a larger cooperative group or consortium. The key to collaborative success is to identify 'win-win' situations for collaboration and to highlight the possible pitfalls of not participating. Benefits that can arise through collaboration range from co-authorship, exchange of technical expertise, exchange of raw data or results between partners, accessing expertise or equipment unavailable to an individual lab, shared costs or other resources and the possibility for networking and having

the opportunity to offer additional services. Implementing these benefits can sometimes be challenging as different institutions may also have their own policies, with different values, preferences and interpretation of legislation which can lead to complexity in discussions regarding the terms of contracts and can be very time-consuming. Therefore, having dedicated support by way of both technological platforms and staff to assist with these interactions is of great benefit and foster trust and collaboration between stakeholders.

Conclusions

By its very nature, biomarker research is interdisciplinary. Now we can expect the contributions of surgeons, pathologists, molecular biologists, biobankers, statisticians, bioinformaticians and imaging experts in addition to clinical expertise. Therefore, coordination and management of both the processes and the various stakeholders involved is an essential requirement particularly to integrate the right expertise at the right moment during the study development and often at very early stage at the concept development. New models of multidisciplinary review committees are being implemented to reflect this shift in the environment. Harmonisation, collaboration and networking efforts are emerging and are now starting to be embraced by the community as it is increasingly recognised that this is needed in order to allow efficient translation of biomarkers to the clinic, to facilitate financial stability and to enable more rapid progress in biomarker research and can be implemented through appropriate translational research infrastructure and access to expertise.

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Biomarkers of Endometrial Cancer

Nicholas Galazis

Abstract EC is one of the commonest cancers worldwide, and its incidence is increasing particularly in the developed world. A patient usually presents with suspicious symptoms (typically PMB) and undergoes a range of investigations and treatment before a definitive diagnosis of EC is made (TVS, hysteroscopy and endometrial biopsy, CT/MRI, surgery for diagnosis, treatment and further staging).

Biomarkers have the potential to help screening, diagnosing and staging the disease and could complement conventional means. At the moment, biomarker utilisation and research are more relevant in facilitating staging of EC and thus guiding treatment and aiding prognosis. Biomarker utilisation in screening and diagnosis is much less developed.

Keywords Endometrial cancer • Biomarker • Screening • Diagnosis • Staging • Prognosis

Introduction

Epidemiology

According to the World Health Organisation, endometrial cancer (EC) is the seventh most common cancer among women worldwide [1]. The incidence varies among different regions, with ten times higher incidence in developed countries compared to developing or less developed countries [2]. In fact, EC occurs in 10–20 per 100,000 women annually, making it the commonest malignant tumour of the pelvis [2].

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The International Federation of Obstetrics and Gynaecology (FIGO) gives the following staging [3]:

- Stage I EC is confined to the corpus uteri:
  - IA confined to endometrium with no or less than half myometrium invaded
  - IB invasion equal to or more than half of myometrium
- Stage II involves the corpus with invasion into the cervical stroma but has not extended outside the uterus.
- Stage III has local or regional spread beyond the uterus:
  - Stage IIIA is invasion of serosa or adnexa or positive peritoneal cytology and possibly more than one of these.
  - Stage IIIB is vaginal or parametrial metastases.
  - Stage IIIC is metastases to pelvic (IIIC1) or para-aortic (IIIC2) lymph nodes or both.
- Stage IV is involvement of the bladder or bowel mucosa or distant metastasis:
  - Stage IVA is involvement of bowel or bladder mucosa.
  - Stage IVB is distant metastases including nodes in the abdomen or inguinal region.

Approximately 72% of EC cases are FIGO stage I at diagnosis, 12% are stage II, 13% are stage III, and 3% are stage IV [4, 5]. The overall survival of patients affected with EC is about 80% and this depends on the FIGO stage [3].

Aetiology and Risk Factors

Despite significant research into the biochemical mechanisms and pathophysiology of EC, the precise aetiology is unknown [6]. EC rarely presents before the age of 40, and more than 80% of cases occur in postmenopausal women [7].

About 5–10% of the cases of EC have a hereditary basis, with hereditary non-polyposis colorectal cancer (HNPCC or Lynch syndrome) being the most common cause. In fact, women with HNPCC have a higher lifetime risk of developing EC than developing colorectal cancer (42% versus 30%) [8]. More than 90% of EC cases occur sporadically [2]. Risk factors include unopposed oestrogen stimulation of the endometrium such is the case in women suffering from polycystic ovary syndrome (PCOS), obesity, diabetes mellitus and oestrogen-secreting tumours [2, 9].

Nulliparity is also a risk factor for EC as well as the use of tamoxifen for the treatment for women with breast cancer [10, 11].

The Dualistic Model

EC is commonly classified into two types. Type 1 tumours (about 80%) are endometrioid carcinomas arising in a background of hyperplasia in obese women [12]. These tumours are usually low grade, oestrogen related and follow a more

favourable course. In contrast, type 2 tumours (about 20%) are non-endometrioid (predominantly serous and clear cell) carcinomas arising in endometrial polyps or from precancerous lesions in the vicinity of an atrophic endometrium. These tumours are high grade, not oestrogen related, often invade the myometrium and (lymph) vascular spaces and have a high mortality rate [13]. At the time of operation, about one in every ten clinical stage I ECs has lymph node metastases, most commonly at pelvic lymph nodes, sometimes associated with para-aortic lymph node involvement [14]. The latter represent a more aggressive disease stage, and it is an independent predictor of poor outcome [15].

Clinical Presentation

Typically, EC presents as postmenopausal bleeding (PMB). In premenopausal women, it presents as menorrhagia, intermenstrual or postcoidal bleeding. EC is usually diagnosed early so women rarely present with systemic symptoms of malignancy like weight loss, tiredness or malaise.

Investigations

Transvaginal Ultrasound

Transvaginal ultrasound (TVS) is an appropriate first-line procedure to identify which women with PMB are at higher risk of EC. The mean endometrial thickness in postmenopausal women is much thinner than in premenopausal women; therefore thickening of the endometrium may indicate the presence of pathology. In general, the thicker the endometrium, the higher the likelihood of important pathology, that is, EC. In the UK, the endometrial thickness threshold is 5 mm which provides adequate sensitivity without excessive false-positive rates in most women and a false-negative rate of 0.25–.50% [16]. European guidelines have a lower threshold (3–4 mm), but this leads to greater numbers of biopsies [17]. Some pathology may be missed; therefore, hysteroscopy and biopsy should be carried out in cases where endometrial thickness is below the threshold if there is a high clinical suspicion [17].

Hysteroscopy and Endometrial Biopsy

A definitive diagnosis in PMB is made by histology. Biopsy can be taken during hysteroscopy performed under local or general analgesia.

Staging

Once histological diagnosis is established, CT of chest, abdomen and pelvis as well as MRI of pelvis should be performed to assess the extent of disease. Further staging is performed intraoperatively which includes exploration of the pelvis and abdomen with biopsy of any suspicious lesions, total abdominal or laparoscopic hysterectomy (TAH/TLH), bilateral salpingo-opherectomy (BSO) and, where appropriate, complete pelvic and/or para-aortic lymphadenectomy [18].

Treatment

This depends upon the stage [19].

- Stage I requires total abdominal hysterectomy with bilateral salpingo-oophorectomy. The role of lymphadenectomy is debated [18].
- In stage II there should be radical hysterectomy with systematic pelvic node clearance. Para-aortic lymphadenectomy may also be considered. Lymphadenectomy is important for staging and as a guide for adjuvant therapy.
- Stages III and IV are best treated with maximal de-bulking surgery. Although there is no conclusive evidence, a combination of surgery, radiation and chemotherapy (usually with doxorubicin).

Molecular Biology and Genetics of EC

The endometrium undergoes structural modification in response to fluctuations of oestrogen and progesterone during the menstrual cycle. Long-lasting unopposed oestrogen exposure leads to endometrial hyperplasia, which increases the chance of development of type 1 EC. The molecular basis of this process is still not known, since the involvement of only a minority of factors is reproducible [20]. Aside from their morphologic and clinical features, type 1 and type 2 ECs are further distinguished by genetic alterations [21].

In general, the development of cancer is characterised by self-sufficiency in growth signals, insensitivity to growth inhibition, evasion of apoptosis, angiogenesis, invasion and metastasis [22]. Understanding pathogenesis at the molecular level is essential in identifying biomarkers for successful targeted therapies.

Type 1 (Endometrioid) EC

The most frequent genetic alteration mainly affecting type 1 EC involves the PTEN gene—a tumour suppressor [23]. PTEN, located at chromosome10q23, encodes a protein (phosphatase and tensin homolog, PTEN) with tyrosine kinase. PTEN has

been reported to be altered in up to 83% of type 1 EC and 55% of precancerous lesions [23]. PTEN inactivation is caused by mutations that lead to a loss of expression and, to a lesser extent, by a loss of heterozygosity. Thus, loss or altered PTEN expression results in aberrant cell growth and apoptotic escape. Loss of PTEN is furthermore probably an early event in endometrial carcinogenesis, as evidenced by its presence in precancerous lesions, and is likely initiated in response to known hormonal risk factors [23]. Its expression is highest in an oestrogen-rich environment. In contrast, progesterone promotes involution of PTEN-mutated endometrial cells. These observations are consistent with the well-documented clinical effects of progesterone-mediated suppression and resolution of invasive EC and its precursors [24]. PTEN mutation is well documented in endometrial hyperplasia with and without atypia [25].

Mutations in PIK3CA may contribute to the alteration of the phosphatidylinositol 3-kinase (PI3K)/AKT signalling pathway mainly seen in type 1 EC [26]. PIK3CA gene mutations occur in 24–39% of the cases of type 1 EC and frequently coexist with PTEN mutations [27]. PIK3CA mutations have been associated with adverse prognostic factors such as high-grade and myometrial invasion [27].

The accumulation of sequence changes in DNA segments, which occurs because of inactivation of intranuclear proteins constituting the mismatch repair system, is known as microsatellite instability (MSI) [27]. MSI has been demonstrated in 20% of sporadic type 1 EC [26]. Microsatellites are short segments of repetitive DNA bases that are scattered throughout the genome. Inactivation of MutL protein homolog 1 (MLH1), a component of the mismatch repair system, is a common event in type 1 EC. This alteration occurs through hypermethylation of CpG islands in the gene promoter, a process known as epigenetic silencing [27]. MSI and abnormal methylation of MLH1 are early events in endometrial carcinogenesis and have also been described in precancerous lesions [28].

Other genetic alterations in type 1 EC include mutations of K-ras and beta-catenin genes [26].

Type 2 (Serous and Clear Cell) EC

The most common genetic alteration in serous EC is in p53, the tumour suppressor gene. This occurs in up to 90% of serous EC [29]. The p53 gene is located on chromosome 17 and is important in preventing the propagation of cells with damaged DNA. The exact mechanism behind the cause of this mutation is still unclear. It is postulated that mutation in one allele occurs early during the development of serous carcinoma, and loss of the second normal allele occurs late in the progression to carcinoma [29].

Other frequent genetic alterations in type 2 ECs are inactivation of p16 and overexpression of HER-2/neu [30]. P16 inactivation was found in 45% of serous carcinomas and some clear cell cancers. The p16 tumour suppressor gene is located on chromosome 9p21 and encodes for a cell cycle regulatory protein. Thus, inactivation of p16 leads to uncontrolled cell growth [30].

Diagnostic/Screening Markers

EC is detected after pathology assessment of uterine aspirates, hysteroscopy-guided biopsies and curettage. Although these methods are considered the gold standard for screening, they still have some limitations and drawbacks [31]. First, they may cause significant discomfort. Second, as tools for diagnosis, they have only a moderate ability to predict the final pathology, and third, they require a trained pathologist for interpretation.

A study by Colas et al. compared gene expression screening on 52 carcinomas and 10 normal tissues to identify potential biomarkers [32]. These were further validated in an independent series of 19 tissue samples by RTqPCR and on 50 carcinoma and non-carcinoma uterine aspirates [32]. A panel of potential genes differentially expressed was identified (ACAA1, AP1M2, CGN, DDR1, EPS8L2, FASTKD1, GMIP, IKBKE, P2RX4, P4HB, PHKG2,PPFIBP2, PPP1R16A, RASSF7, RNF183, SIRT6, TJP3, EFEMP2, SOCS2 and DCN) which correlated to their expression in the corresponding primary endometrial tumours [32]. The authors proposed that such a minimally invasive and highly sensitive and specific method for the identification of EC which has the potential to increase patient comfortability as alternative methods of diagnosis is based in more invasive techniques [32]. It could also provide a molecular tool for supporting pathologist decision and hence help gynaecologists to reduce the number of unnecessary hysteroscopies. Furthermore, among the potential clinical applications for these newly discovered molecular biomarkers could be a screening programme within high-risk populations designed to improve the early detection of EC [32]. Large validation studies need to be conducted first before such results are translated in clinical practice.

DNA methylation is notable because of its early occurrence in carcinogenesis, stability and detectability using highly sensitive and specific assays [33]. Based on the hypothesis that candidate DNA methylation markers demonstrate low values in benign tissues, large differences between carcinomas and benign tissues and highly statistically significant differences by disease status, Wentzensen et al. were able to identify an eight-marker panel obtained from endometrial brushings with substantial discrimination (ADCYAP1, ASCL2, CDH13, HS3ST2, HTR1B, MME, NPY, SOX1) [33]. These findings provide a proof of principle that it may be possible to develop diagnostic molecular testing as an adjunct to the classification of endometrial biopsies or brushings performed to assess suspicious vaginal bleeding [33]. What is more, this test could enable triage patients with carcinoma, while reducing overtreatment of innocuous lesions [33]. This could be particularly important among women with limited health care access as rapid identification of carcinomas may increase chances of cure and reduce the need for more aggressive treatment secondary to disease progression, whereas ruling out high-risk lesions could allow many women to safely opt for conservative management [34]. Again, validation of this biomarker panel in large prospective studies is imperative before the results can be applied in practice.

Higher serum CA 125 levels correlate with the extrauterine disease and advanced cases and are used as a marker to evaluate prognosis and recurrence in EC (see section below on Prognosis and Staging). However, a CA 125 level greater than 35 U/mL is not useful in diagnosing early stages of EC [35]. Moore et al. have proved that serum HE4 is elevated in all stages of EC and is more sensitive in early-stage cancer compared to CA 125 [36]. Although there is sufficient evidence in regard to the accuracy of HE4 for the diagnosis of EC, there is currently not enough data to estimate its value in clinical practice [35]. Such quantification warrants further large-scale studies. Finally, there is evidence that patients with EC have significantly different expression patterns of several serum biomarkers as compared to healthy controls with a high sensitivity (98.3%) and specificity (98.0%) [37].

In conclusion, the role of biomarkers in the screening and diagnosis of EC is still in its infancy, and further studies are needed to validate these promising findings before they are translated to clinical practice.

Prognostic/Staging Biomarkers

Tissue Biomarkers

Expression of p53 protein and/or p53 gene mutations have been detected in 7–43% of EC and have been associated with advanced stage, high grade, deep myometrial invasion, type 2 histology, lymph node metastasis and, ultimately, lower survival compared with EC patients without p53 alterations [38–43].

PTEN mutations are related to early stage, low rate of p53 overexpression and longer survival in women with EC [44]. On the other hand, Steinbakk et al. failed to evidence any prognostic relevance for PTEN status in curettages from patients with FIGO stages I–II type 1 EC [43]. Therefore, loss of PTEN function did not appear to impact on survival of patients with early disease, but it was associated with a better clinical outcome in those with advanced or recurrent disease [45].

MSI, which is the hallmark of defects in DNA mismatch repair genes, occurs in 11–45% of type 1 EC [45–47]. Whereas MSI is an independent predictor of a favourable outcome in colorectal cancer [48], conflicting data emerge from the literature as far as the prognostic relevance of MSI in type 1 EC is concerned [49].

Alterations in β-catenin expression have been reported both in type 1 EC and atypical hyperplasia and therefore appear to represent an early event in endometrial carcinogenesis [26]. Saegusa et al., who assessed 199 cases of type 1 EC, found a significant association between β-catenin mutations and low-grade histological malignancy ($p = 0.048$), as well as between β-catenin mutations and lack of lymph node involvement [50].

K-ras mutations which are most commonly seen in type 1 EC have been associated with lymph node metastasis and poor survival [26, 51, 52]. For example, Mizuuchi et al. investigated 49 cases and concluded that the presence of K-ras

mutations was an independent predictor of unfavourable clinical outcome ($p = 0.034$) after adjusting for tumour stage, depth of myometrial invasion and patient age [52].

Vascular endothelial growth factor (VEGF) is an important endothelial cell mitogen that acts through specific receptors, namely, flt-1 and flk-1/KDR [53]. In EC, an increase in VEGF expression has been often associated with advanced tumour stage [54], high tumour grade [55], deep myometrial invasion [56], lymphovascular space involvement [54] and lymph node metastases [54].

The proportion of aneuploid tumours among EC ranges from 16 to 28% and significantly correlates with old age at diagnosis, type 2 histology, high tumour grade and lymph node involvement [57–60]. In most studies patients with aneuploid tumours have significantly poorer survival at multivariate analysis, after adjusting for the common clinical-pathological variables [57, 59, 60]. In fact, some authors have suggested including DNA ploidy among criteria for the selection of high-risk patients who might benefit from adjuvant treatment [58, 59].

Both HER2 overexpression and amplification have been linked to poor prognosis and survical in EC [61–63]. Following the successful development of targeted therapy against HER2 in breast cancer, reports on HER2 overexpression have sparked considerable interest for a potential novel HER2-based therapy in EC. Trastuzumab (Herceptin, Genentech, San Francisco, California), a humanised monoclonal immunoglobulin (Ig) G1 antibody against HER2/neu, is now Food and Drug Administration (FDA) approved in the treatment of HER2-overexpressing breast cancer and HER2-overexpressing metastatic gastric or gastroesophageal junction adenocarcinoma [61]. In vitro studies have demonstrated that trastuzumab results in antibody-dependent cellular cytotoxicity in the range of 25–60% against HER2-overexpressing uterine serous carcinoma which can be augmented by both IL-2 and simultaneous administration of the heterodimerization inhibitor pertuzumab (Omnitarg, Genentech) [61].

Hormone receptor status has consistently been shown to be a relevant prognostic marker that could also influence the choice of treatment for metastatic disease, due to higher response rates reported for hormone-receptor-positive tumours [64]. The presence of steroid receptors correlates with low-grade, type 2 histology as well as favourable outcome in many studies [65, 66]. Hormone receptor status in curettage and hysterectomy specimens has been reported to be highly correlated with favourable prognosis and with good to very good reproducibility for pathological staining assessment [65]. On the contrary, loss of oestrogen and progesterone receptors in curettage specimens has been significantly associated with aggressive phenotypes and poor survival in patients with EC [66].

Serum Biomarkers

Elevated serum CA 125 levels (>35 U/mL) have been found in 11–34% of patients with EC [61–64]. Preoperative serum CA 125 concentrations correlate with stage [67–71], depth of myometrial invasion [65–67], tumour grade [69, 71, 72], cervical invasion [73], peritoneal cytology [71, 73] and lymph node status [67, 71, 73]. A

preoperative test that could accurately recognise nodal disease would prevent both overtreatment (i.e. unnecessary pelvic and para-aortic lymphadenectomy) and undertreatment (i.e. withholding complete lymphadenectomy or adjuvant postoperative treatment to patients with lymph node metastases). However, the optimal cutoff value has not been determined yet, and correlations between the levels of CA 125 in serum and lymph node metastases remain inconsistent, warranting further research in this field [74].

Several studies have investigated whether serum CA 125 assay may provide additional information for the identification of those patients with high risk of subclinical extrauterine spread who need a lymphadenectomy [67, 71, 73]. Scambia et al. found CA 125 levels >65 U/mL in 22% of patients with negative lymph nodes compared to 58% of cases with histologically proven positive nodes ($p = 0.022$) [67]. Sood et al. observed that preoperative serum CA 125 > 65 U/mL was the strongest predictor of extrauterine disease with a risk ratio of 6.5 (95% CI = 2.5–17.1) [71]. Other authors confirmed that serum CA 125 level was an independent risk factor for lymph node involvement [73, 74].

Serum CA 153 levels are elevated in 24–32% of patients with EC and correlate with tumour stage [67, 69]. Scambia et al. detected CA 153 levels >30 U/mL in 47% of patients with stage III disease compared with 18% of those with stage I–II disease ($p = 0.01$) and found a significant relationship between serum CA 153 positivity (>30 and >50 U/mL) and shorter survival ($p = 0.0004$ and $p = 0.00025$, respectively) [67].

Conclusions and Future Approaches

Biomarkers have the potential to help screening, diagnosing and staging the disease and could complement conventional means. At the moment, biomarker utilisation and research are more relevant in facilitating staging of EC and thus guiding treatment and aiding prognosis. Biomarker utilisation in screening and diagnosis is much less developed.

There are important limitations that need to be overcome in the future to allow adequate implementation of new biomarkers to guide clinical care in EC. Suggestions for future research include [64]:

1. Sufficiently sized, population-based biomarker studies linked to state-of-the-art clinically and histopathologically annotated patient series.
2. The test criteria applied for new surgical staging procedures by lymphadenectomy should be better standardised, and figures for reproducibility, sensitivity and negative predictive value for the procedure should be established.
3. Introduction of new imaging methods and biomarkers for test development needs to meet strict standards for reproducibility and test quality before incorporation into stratification schemes that define target populations.
4. Studies of new potential markers need to be done in a prospective mulitcentre setting to document their performance in a routine clinical setting.

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Biomarker Development in Chronic Inflammatory Diseases

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Abstract Chronic inflammatory diseases, such as inflammatory bowel disease—namely, Crohn's disease and ulcerative colitis—psoriasis, multiple sclerosis, rheumatoid arthritis, and many others affect millions of people worldwide, causing a high burden of disease, socioeconomic impact, and healthcare cost. These diseases have common features including autoimmune pathogenesis and frequent co morbidity.

The treatment of these chronic inflammatory diseases usually requires long-term immunosuppressive therapies with undesirable side effects. The future of chronic inflammatory disease prevention, detection, and treatment will be greatly influenced by the use of more effective biomarkers with enhanced performance. Given the practical issues of collecting tissue samples in inflammatory diseases, biomarkers derived from body fluids have great potential for optimized patient management through the circumvention of the abovementioned limitations.

In this chapter, peripheral blood, urine, and cerebrospinal fluid biomarkers used in chronic inflammatory conditions are reviewed. In detail, this chapter reviews biomarkers to fore used or emerging to be used in patients with chronic inflammatory conditions. Those include inflammatory bowel diseases, chronic inflammatory

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conditions of the liver, biliary tract, pancreas, psoriasis, atopic disease, inflammatory skin diseases, rheumatic diseases, demyelination, and also the chronic inflammatory component of various other diseases in general medicine—including diabetes, cardiovascular disease, renal disease, and chronic obstructive pulmonary disease.

Development of personalized medicine is closely linked to biomarkers, which may serve as the basis for diagnosis, drug discovery, and monitoring of diseases.

Keywords Biomarkers • Chronic inflammation • Inflammatory bowel diseases • Neurology • Dermatology • Rheumatology • Diabetes • Liver • Pancreas • Medicine

Introduction

As personalized medicine is becoming an integral component of modern healthcare, the development of biomarker-based clinical tests emerges as a key challenge. In the conventional setting, chronic inflammatory diseases are assessed by clinical activity indices that measure clinical symptoms or by radiologic or endoscopic indices that measure tissue inflammation. Disease subtypes are often defined on the basis of medical history, physical findings, imaging parameters, serum markers, as well as endoscopic and histopathological characteristics of the disease. Noninvasive or minimally invasive diagnostic procedures present less burden and risk to patients than, for example, invasive biopsy sampling. They may thus support clinical decision-making by providing information on disease status and prognosis. The requirements of these applications cannot always be met by a single biomarker class.

A comprehensive biomarker approach is needed, given its ultimate significance for clinical application. This strategy could lead to an optimized management of chronic inflammatory diseases with existing drugs and may facilitate the development of novel, more effective therapies. Chronic inflammation is a component of many disorders that are discussed as per the system involved. Major disorders with chronic inflammation involve many diseases including those of the digestive tract, liver, and cardiovascular and nervous systems. Diseases such as diabetes and rheumatoid arthritis are also characterized by inflammation. In addition, rejection of allografts also involves chronic inflammatory immune mechanisms, and there is an interest in finding predictive biomarkers of organ rejection after transplantation.

This chapter provides an overview of the field of biomarkers in the peripheral blood, urine, or cerebrospinal fluid (CSF) that are currently used or can be easily used in combination with other clinical facilities to diagnose, treat, and tailor therapy in patients with chronic inflammatory diseases.

Biomarkers in Inflammatory Bowel Diseases

For patients diagnosed with inflammatory bowel diseases (IBD) including Crohn's disease (CD) or ulcerative colitis (UC), several laboratory markers have been investigated for diagnosis and differential diagnosis of IBD as well as for assessment of disease activity and risk of complications, prediction of relapse, and monitoring the effect of therapy [1, 2].

We can differentiate the biomarkers in three major categories: serological, fecal, and other biomarkers. Each category includes other subcategories, for instance, serological biomarkers are differentiated in acute-phase reactants, cytokines, and others [Table 1].

Serological biomarkers are measurable substances in body fluids (blood), whose application is cheaper, less laborious, less invasive, and more objective compared to the endoscopy-/biopsy-based approach [3]. Serological acute-phase proteins are those whose plasma concentration increases (positive acute-phase proteins) or decreases (negative acute-phase proteins) by at least 25% during inflammatory disorders [1]. It is widely accepted that the concentrations of multiple components of the acute-phase response do not increase uniformly in all patients with the same illness, although the serological acute-phase proteins commonly increase together.

As serum markers can be elevated in a variety of conditions, fecal markers of inflammation would be more specific for IBD, in absence of enteric infection [4]. Fecal biomarkers are valuable in their specificity to the gastrointestinal tract. They include a heterogeneous group of substances that either leak from or are generated by the inflamed intestinal mucosa [1]. In the setting of mucosal inflammation in patients with IBD, inflammatory proteins (protein cytokines and markers C of neutrophil activation), leukocyte products, and leukocytes themselves leak from a permeable mucosa. Fecal microbiota appear to be attractive biomarkers in IBD, since they provide a noninvasive way to directly monitor changes in the intestinal environment associated with mucosal inflammation. However, they do not necessarily play a causative role in disease. The ability of the organism to compete in the altered intestinal environment is associated with the appearance or disappearance of a microbial group in disease [5].

Table 1 Routine biomarkers used in inflammatory bowel disease

Serological	Acute phase reactants	CRP, ESR, platelets, orosomucoid, ASCA, pANCA, ALCA, ACCA, AMCA, anti-OmpC, anti-flagellin antibodies, anti-I2	
	Cytokines	Interleukins	Others
		IL-1, IL-2, IL-6, IL-10, IL-17, IL-23	TNF-a
Fecal	Serum proteins	Calprotectin, lactoferrin	

Serological Biomarkers of Acute Phase Response

C-reactive Protein

C-reactive protein (CRP), an acute-phase protein, is produced as an acute-phase reactant predominantly in the liver, in response to a variety of acute and chronic inflammatory conditions, and is an important component of the innate immune system [1, 4, 6]. CRP is produced mainly by hepatocytes in response to stimulation by interleukin (IL)-6, and to a lesser extent in response to TNF-a and IL-1b, which are produced at the site of inflammation [1, 4].

CRP is an easy and reliably measured biomarker across diagnostic laboratories and has a short plasma half-life of 19 h [6]. CRP production is rapidly upregulated, in the presence of an acute-phase stimulus [4]. Within 24–48 h the increase may be 500 to 1000-fold higher than under basal circumstances. Inversely, to its increase, the reduction of CRP may be similarly rapid, as the acute-phase response subsides, with a fall from peak with a half-time of 48 h [1]. Once the stimulus disappears, CRP concentrations quickly decrease due to CRP's short half-life. Hence, this makes CRP a valuable marker to detect the activity of IBD [4]. CRP is an objective marker of inflammation correlating well with disease activity in CD. Increased CRP levels are associated with better response rates, whereas normal CRP levels predict high placebo response rates in clinical trials [2]. The same increasing trend can be observed in UC, although CRP levels are generally lower than in CD [4]. Several studies have identified increased levels of CRP in nearly 100% of patients with CD and approximately 50% of those with UC [7].

CRP is the most sensitive biomarker compared to other biomarkers of inflammation in adult population for the detection of IBD and is also used in screening patients with gastrointestinal symptoms. The sensitivity of CRP in discriminating CD from irritable bowel syndrome ranges between 70 and 100% and the sensitivity in UC between 50 and 60% [1]. Hence, CRP is a valuable biomarker in sorting out active CD from functional bowel disorders [6]. Moreover, since CRP levels tend to be higher in CD than in UC, CRP might be used to differentiate both types of IBD [1]. However, the relatively low sensitivity of the CRP test in detection of UC prohibits the use of this marker alone to identify patients with symptoms compatible with IBD, without further evaluation [7].

CRP distinguishes between active and quiescent IBD and trends with mucosal healing [6]. Various studies have been conducted, regarding CRP levels and their correlation to different clinical courses [4]. A retrospective analysis of Mayo Clinic data has associated moderate to greater clinical disease severity, active disease on colonoscopy, and histological severe inflammation with an elevated CRP, while 51% of UC patients with active disease also had an elevated CRP. In a follow-up investigation, among patients with symptoms of active CD and an elevated CRP, 86% exhibited evidence of inflammation at colonoscopy [4, 6]. This suggests that CRP has the ability to predict active mucosal inflammation. Across studies, however, it has been showed that CRP (in both UC and CD patients), when compared to

calprotectin and lactoferrin, is less sensitive and has a lower correlation with mucosal inflammation as shown by endoscopy [6]. Studies in patients with CD, with clinically inactive disease and elevated CRP, have demonstrated that the latter had higher chance of relapse in the following 2 years than those with normal CRP. In UC patients, CRP elevation was significantly correlated with severe clinical activity and active disease by means of colonoscopy but not with histological inflammation [4]. Yet, some patients have had persistent, normal levels of CRP despite active disease [7]. These patients often exhibit exclusive ileal disease and low body mass index [4]. For these patients, CRP will not be a useful marker for differentiation of quiescent from active disease [7]. Generally, the value of CRP as a predictor of relapse is controversial, with some studies considering it an accurate predictor and others not. A combination of CRP and erythrocyte sedimentation rate (ESR) has also been used to predict relapse in patients with CD [4].

Erythrocyte Sedimentation Rate

ESR is the rate at which red blood cells (RBC) migrate through the plasma over the period of 1 h [6]. The ESR determination is a commonly performed laboratory test and reflects the changes in the various acute-phase proteins [1]. The test measures the distance that erythrocytes have fallen after 1 h in a vertical column of anticoagulated blood under the influence of gravity [1]. ESR is not rapidly responsive to changes in clinical status, as the concentrations of many serum proteins vary and some have long half-lives [4]. ESR determinations have been shown to be satisfactory monitors of acute-phase response to disease after the first 24 h [1]. Therefore, ESR is less suited to detect changes in disease activity, and it fails to exhibit the real clinical course, as it may take several days to decrease even when rapid clinical improvement occurs. Hence, ESR is an indirect, crude, but fairly rapid assessment of the general acute-phase response [1, 4, 6].

Except from erythrocytes morphology and plasma constituents, such as immunoglobulins, ESR is influenced by several factors including age, gender, anemia, blood dyscrasias, and pregnancy [4, 6]. Although it is still widely used, especially as a biomarker of IBD activity, its usefulness has decreased as new methods of evaluating disease have developed [1, 6]. In UC there is a good correlation between ESR and disease activity. In CD the ESR appears to be a less accurate measure of disease activity [4]. The increase of ESR in increasing disease activity correlates more with colonic disease and does not reflect the disease activity in the small bowel [4]. Compared with CRP, during the first 24 h of inflammation, ESR will peak less rapidly, and may take days to decrease, even if the inflammation has subsided. However, CRP tests are costly, frequently unavailable, and more time-consuming to perform than the ESR [1].

Platelets

Besides their function, which is to stop bleeding, platelets also have a recognized role in inflammatory processes [6]. Therefore, platelet count correlates with disease activity in IBD [4]. The high platelet number correlates well with disease severity and may persist even after bowel resection in IBD patients [1]. Increased platelet concentration in the blood circulation of IBD patients is not yet understood, but is associated with inflammation, as a nonspecific response [1]. Thus, their relationship to IBD pathophysiology demands further investigation [6].

Platelet count is a potential method for the distinction of IBD from infectious diarrhea [1]. In active IBD, the platelet count may be elevated, while the mean platelet volume is low [6]. Causes of this occurrence are unknown, but there might be an association with the thrombopoiesis disturbance often observed in the early stages of systemic inflammatory processes, as occurs in CD and UC [1]. According to studies, in more than 30% of IBD patients, spontaneous platelet aggregation takes place independently of disease severity [1]. While the normal level varies considerably, in practice, the platelet count is routinely available in patients and, if elevated, may alert the clinician about ongoing inflammation [6].

Serological Markers with Restricted Clinical Use in IBD

Orosomucoid

Orosomucoid or alpha-1-acid glycoprotein is an acute-phase plasma glycoprotein, which is synthesized primarily in hepatocytes [6]. The levels of circulating orosomucoid have been shown to correlate with disease activity of IBD as assessed by standard disease activity indices [4, 6]. However, the long half-life (5 days) diminishes its usefulness in practice [6]. Therefore, orosomucoid does not appear to be a useful marker for screening health populations or for distinguishing between patients with inflammatory versus functional disorders [1].

Anti-*Saccharomyces cerevisiae* Antibodies

The most prominent member of anti-glycan antibodies is *Anti-Saccharomyces cerevisiae antibodies (*ASCA) [8], which are formed both as IgG and IgA antibodies [9]. They are believed to interact with mannose residues on mannan in the cell walls of *S. cerevisiae*. More specifically, the major antigen targeted with ASCA antibodies is a mannan, a cell wall glycoprotein, the 200 kDa phosphopeptidomannan (PPM), of the common baker's or brewer's yeast *Saccharomyces cerevisiae* [1, 7, 8]. In particular, the Su1 strain of *S. cerevisiae* used in beer brewing and mannotetraose has been labeled as the most vital polysaccharide epitope within PPM [8].

The production of these antibodies is poorly understood [9]. Therefore, three theories have been proposed regarding the widespread distribution of oligomannosides: The first theory is that dietary yeasts or yeasts that colonize the digestive tract activate the production of ASCA antibodies. The second theory concerns the epitopes shared by other microorganisms (*Mycobacterium* species). The third one assumes that there are structural homologies between *S. cerevisiae* oligomannosides and oligomannosides expressed on human glycoconjugates as autoantigens or neoautoantigens [8].

ASCA are widely used as biomarkers for the discrimination among patients with CD versus those with UC [7, 8]. Increased titers of ASCA were reported to identify CD with high levels of specificity (96–100%) but low sensitivity (approximately 50%), while both IgA and IgG antibodies are formed [7, 8]. Indirect immunofluorescence (IIF) and standardized enzyme-linked immunosorbent assays (ELISA) are the two main methods used for the detection of these antibodies [8]. Moreover, the titers of these antibodies do not seem to correlate with disease activity, and ASCA levels appear to be stable for long periods. In fact, patients with CD have been reported to have still high rates of ASCA, though their last outbreak of CD was 20 years ago and their markings on gastroscopy, colonoscopy, and histology were normal [9]. Interestingly, the results vary between different populations, as the sensitivity of ASCA IgA is lower in Japanese and Chinese CD patients when compared to Caucasian CD patients [1]. This observation suggests that several distinct genetic determinants and/or environmental risk factors may influence the ASCA response [1].

Yet the question still remains, do high rates of ASCA antibodies indicate a predisposition to IBD and are they genetic markers or not? Numerous studies have been performed; in one study, ASCA antibodies were detected in 20–25% of healthy first-degree relatives of patients with CD. Another study showed detectable levels of ASCA in 31% of patients long before they were diagnosed with CD. For them the maximum frequency of antibodies was recorded 36 months before diagnosis [9]. These studies indicate that ASCA growth happens before or during early stages of disease. Hence, we assume that ASCA are likely produced in the context of early development of the disease, rather than as genetic markers in childhood. Further study needs to be done in order to conclude whether ASCA antibodies serve as indicators of future disease. For now, the initial event leading to IFPE is still unclear, but it is of great interest [9].

Anti-neutrophil Cytoplasmic Antibodies

Anti-neutrophil cytoplasmic antibodies (ANCAs) are a group of autoantibodies, mainly of the IgG type, against antigens in the cytoplasm of neutrophil granulocytes (the most common type of white blood cell) and monocytes. They are detected during a blood test in a number of autoimmune disorders but are classically associated with small-vessel systemic vasculitis, so called ANCA-associated vasculitides, such as Wegener granulomatosis, Churg-Strauss syndrome, microscopic polyangiitis,

and its renal-limited variant (pauci-immune necrotizing and crescentic glomerulonephritis) [6, 8, 10]. In addition, ANCAs are found in other chronic inflammatory disorders, most notably in rheumatoid arthritis and in UC [1]. Serum levels of ANCA are used for the purposes of diagnosis and prognosis and in monitoring of inflammatory activity [1, 8].

In vasculitis, ANCA antibodies target different proteins, usually located in the lysosomes of monocytes and in the azurophilic granules of neutrophils. The most commonly employed method in use as a screening method for ANCAs is indirect immunofluorescence (IIF) on normal peripheral blood neutrophils [8]. But for diagnostic purposes, enzyme-linked immunosorbent assay (ELISA) is used in laboratories to detect ANCAs [8, 10]. According to IIF, there are two types of fluorescence pattern: cytoplasmic granular with central interlobular accentuation (cANCA) and fine homogenous, rim-like staining of the perinuclear cytoplasm (or rim-accentuated fluorescence of the nuclei) designated as the pANCA pattern. Various target antigens of atypical pANCA have been intensively studied in IBD patients, and so far results have been conflicting regarding their potential use in clinical practice as their sensitivity and specificity are less than 60% [8]. It is likely that the target antigen for UC-related atypical pANCA is a complex conformational epitope which comprises the nuclear proteins histone H1, HMG-1, and HMG-2. Nevertheless, since the target antigen for UC-associated pANCA is yet unrecognized, sensitive and specific solid-phase methods cannot be developed [8]. The sensitivity of the IIF assay for UC reaches the 87.2% [8].

Anti-glycan Antibodies

ACCA, ALCA, and AMCA (ACCA, anti-chitobioside carbohydrate IgA antibody; ALCA, anti-laminaribioside carbohydrate IgG antibody; AMCA, anti-mannobioside carbohydrate IgG antibody) are novel anti-glycan antibodies to sugars on the surface of microorganisms. Various studies have taken place that have associated ALCA and ACCA with CD [6]. Interestingly, in a study with CD patients, a rate of 17–28% ALCA and ACCA was found, while the sensitivity ranged between 34 and 44% in CD patients who were ASCA negative [6, 7]. ASMA antibodies also seemed to be positive in 24% of patients with CD who were negative for ASCA and had a lower sensitivity but higher specificity when compared with ASCA. Moreover, ACCA and ALCA antibodies have similarly exhibited low sensitivity but relatively high specificity in CD patients, compared to patients with UC [6, 7]. Other studies have shown that the combination of gASCA, pANCA, and ALCA is more accurate than each individual test separately in distinguishing individuals with IBD (UC or CD) from healthy controls [7].

Antibody to Outer Membrane Porin (Anti-OmpC)

OmpC is a major outer-membrane protein, porin C isolated from *Escherichia coli* [1, 8]. Adherent-invasive *E. coli* has been found in ileal CD lesions, and OmpC is necessary for these organisms to thrive in the gastrointestinal tract [6]. An excessive secretion of OmpC antibodies has been recently reported, with ELISA assay, mainly in CD patients (55%), while it was insignificant in UC patients and in healthy subjects (5–11% and 5%, respectively) [1, 8]. Originally, this protein was identified as a pANCA cross-reactive antigen using the library of colonic bacteria [8]. Anti-OmpC may also be of value in aiding diagnosis of ASCA negative CD patients [1].

Anti-flagellin Antibodies

Flagellin is a protein that arranges itself in a hollow cylinder to form the filament in bacterial flagellum. It is the principal substituent of bacterial flagellum and is present in large amounts on nearly all flagellated bacteria. Flagellin is a common bacterial antigen which plays a vital role in mucosal immune responses, as it is present in most motile bacteria in the gastrointestinal tract and is highly antigenic [8]. Anti-flagellin antibodies can be directed against flagellin (Anti-Cbir1), *Pseudomonas fluorescens*-associated sequence I-2 (Anti-I2), and flagellins A4-Fla2 and Fla-X [6].

Anti-Cbir1

Cbir1 has been identified as an immunodominant colitogenic antigen which was initially identified in the enteric flora of mice and has the ability to induce colitis in immunodeficient mice [6, 8]. Cbir1 is closely related to flagellin from *Butyrivibrio*, *Roseburia*, *Thermagota*, and *Clostridium* species and appears in the *Clostridium subphylum* cluster XIVa of Gram-positive bacteria [1]. Cbir1 has been measured in human sera with the ELISA assay and has shown a high anti-CBir1 IgG reactivity (50%) in CD patients while has only exhibited minor reactivity in UC patients (5–11%) or other inflammatory GIT diseases [6, 8].

Antibody to *Pseudomonas fluorescens*: Associated Sequence I2 (Anti-I2)

The sequence I2 was discovered in 2000, and this DNA was homologous to the ptxR and tetR bacterial transcription factor family, which was isolated from CD colonic lesional mucosa [6, 8]. This suggests that the microorganism expressing the I2 gene product might be related to CD pathogenesis. This sequence derives from *Pseudomonas fluorescens* [8]. Studies using ELISA assays have shown 30–50% IgA seroreactivity against I2 in CD, 10% in UC 36–42% in indeterminate colitis, 19% in patients with other inflammatory gastrointestinal diseases, and 5% in healthy

controls [1, 6, 8]. Thus, the presence of anti-I2 seems to correlate with small bowel perforating disease [1].

Antibodies to Flagellins A4-Fla2 and Fla-X

Flagellins A4-Fla2 and Fla-X are newly identified flagellins. According to data some CD patients are seropositive to them. A 2-year study of 252 patients with CD was carried out which indicated that 76% of these patients had small bowel CD and 59% had antibodies to A4-Fla2 and 57% to Fla-X. Incidentally in a cross-sectional study, antibodies to flagellin A4-Fla2 and Fla-X were found in 29% and 26% of IBS patients, respectively [6]. Anti-flagellin antibodies need further refinement before they can be used in IBD clinical practice and routine diagnostics.

Emerging Serological Markers in IBD: Cytokines

The cytokines are intercellular signaling polypeptides produced by activated cells. In patients with active IBD, the expression of proinflammatory cytokines is markedly increased in the intestinal mucosa, but this increase is not always translated in higher serum concentrations. Some cytokines used as biomarkers are various interleukins (IL-6, IL-10, IL-17, IL-23) and tumor necrosis factor (TNF) and its receptor [1, 4].

Interleukins

Interleukins are detected in blood serum, and the most common noninvasive method of detecting them is ELISA (enzyme-linked immunosorbent assay), a widespread form of biochemical analytical test which uses a subtype and a heterogeneous solid-phase enzyme immunoassay for the detection of a substance, typically an antigen, in a liquid sample [11].

Some of the emerging interleukins, their sources, and biological activity are presented below [Table 2].

TNF-a and TNFa Receptors

Tumor necrosis factor a (TNFa) is produced by activated macrophages and monocytes. Serum levels of TNFa are usually increased in patients with active IBD, though they are not consistently elevated [4]. Hence, TNFa determination is of limited utility as a marker of disease activity in IBD patients [4]. Nowadays, biological therapies targeting TNFα have significantly improved the management of IBD refractory to conventional therapies, thus, turning TNFα into a crucial mediator of

Table 2 Emerging cytokine that could be used as biomarkers in IBD

Interleukin	Sources	Biological activity
Interleukin 1 (IL-1) **Interleukin 1α (IL-1α)** **Interleukin 1β (IL-1β)**	Activated mononuclear macrophages, fibroblasts, dendritic cells, B lymphocytes, NK cells, and epithelial cells	Possess strongly proinflammatory effect. Mediates the inflammatory responses of the host. Stimulates the production of chemokines and acute-phase proteins and activates fever
Interleukin 2 (IL-2) 55–75 kDa	Activated from antigen T cells	Stimulates proliferation of T cells, participates in apoptosis of T cells
Interleukin 6 (IL-6) 26 kDa. Stimulatory factor 2 of the B cells (BSF-2), hybridoma/plasmacytoma growth factor (HPGF), hepatocyte stimulating factor (HSF)	T cells, B cells, several nonlymphocytes including macrophages, stromal cells of the bone marrow, fibroblasts, endothelial cells, and astrocytes	Sets B and T cell functions. Effect in vivo in the process of hematopoiesis. Induction of acute-phase response
Interleukin 10 (IL-10) 35–40 kDa. Inhibitor of cytokine synthesis (CSIF)	Activated subpopulations of CD4+ and CD8+ T cells	Stimulates or enhances the proliferation of B cells, mast cells and thymocytes. In collaboration with the TGF-b stimulates the synthesis and secretion of IgA by B cells of humans. Antagonizes the creation of TH1 subset of T helper cells
Interleukin 17 (IL-17) 28–31 kDa. CTLA-8 (8 antigen associated with cytotoxic lymphocytes)	Mainly CD4+ T cells	Helps hematopoiesis indirectly, stimulating cytokine production by epithelial, endothelial, and fibroblast cell layers. Enhances expression of ICAM-1, thus giving the cells more adhesive ability
Interleukin 23 (IL-23) heterodimer comprising the p40 subunit of IL-12 (35–40 kDa) and the p19 subunit (18.7 kDa)	Activated dendritic cells	An important factor inducing differentiation of TH1 subset of T helper cells. Also induces interferon production by T cells and NK cells and enhances NK activity

this abnormal immune response [11]. The most widely used anti-TNFα agents are infliximab and adalimumab. Infliximab is the best studied anti-TNFα agent and is currently approved in the European Union for adults and children with CD and adults with UC [11]. In CD and UC, infliximab has confirmed efficacy in adults with benefits observed in both clinical remission and mucosal healing. It has also shown similar effectiveness in children with CD [11]. Evidence suggests that early treatment with infliximab may improve the natural course of the disease [11]. Other

cytokines except TNFalpha have been extensively studied, but drug development based on other cytokines is still under way.

Fecal Biomarkers in IBD

Fecal Calprotectin

Calprotectin is a 36 kDa calcium- and zinc-binding protein that represents 50–60% of cytosolic proteins in granulocytes [6, 7]. It has antimicrobial effects and is stable in feces for 1 week [7]. Calprotectin is measured by enzyme-linked immunosorbent assays (ELISA), and its concentration is an indirect measure of neutrophil infiltrate in the bowel mucosa [6, 7]. It is released with cell death or activation, making it a sensitive marker of inflammation [6]. Various studies identify calprotectin as a sensitive marker of activity in CD, which also correlates well with endoscopic and histological activity in UC [4, 7]. Increased levels of fecal calprotectin had a positive predictive value of 81% and a negative-predictive value of 90% for relapse of UC. In patients with CD, the positive predictive value was 87%, and the negative-predictive value was 43% [6, 7]. Its sensitivity and specificity of relapse in both CD and UC ranges approximately at 90% and 83%, respectively. Additionally, the sensitivity and specificity for identification of IBD in adults were 93% and 96%, while, in children, the test confirmed 92% and only 76%, respectively [4, 6, 7]. Despite calprotectin's high sensitivity and specificity, it ought not to be considered as a specific marker of inflammation, since increased levels are also found in neoplasia, polyps, microscopic colitis, allergic colitis, active celiac disease, infections, nonsteroidal anti-inflammatory enteropathy, increasing age, etc. [1, 6]. Relatively high levels of calprotectin are noticed in the stools of normal individuals, and this data is compatible with the hypothesis that in normal individuals most circulating neutrophils migrate through the mucosal membrane of the gastrointestinal tract wall and thereby terminate their circulating life [1].

Fecal Lactoferrin

Another fecal biomarker of inflammation is lactoferrin, an iron-binding glycoprotein found in neutrophil granules, which possesses antimicrobial properties [4, 6]. Fecal lactoferrin is easily quantified using an ELISA specific for human lactoferrin; it is resistant to freeze-thaw cycles and degradation, facilitating its use as a laboratory test [1]. Multiple studies estimated that the lactoferrin test identified patients with IBD with a mean sensitivity of 80% and specificity of 82% [7]. Furthermore, the protein's concentration increases in active IBD, compared to inactive IBD with specificity between 85 and 90%. Fecal lactoferrin levels may rise significantly prior to a clinically evident relapse and thus may be a good marker to predict subsequent IBD flares [4].

Biomarkers in Chronic Inflammatory Diseases of Hepatology

Over the past decade, there has been a renewed enthusiasm for developing noninvasive serum markers or tests to assess the presence and severity of chronic inflammation and fibrosis in chronic liver disease. Although a single marker or test has lacked the necessary accuracy to predict fibrosis, different combinations of these markers or tests have shown encouraging results [12].

Biomarkers of Chronic Viral Infections Affecting the Liver

For hepatitis B virus, the level of HBV DNA in serum or plasma probably reflects the replicative activity of HBV. Various techniques for detection of HBV DNA have been developed, including hybridization assays and PCR. For hepatitis C virus, the introduction of the approved immunoassay EIA has reduced the incidence of HCV transmission via blood transfusion. Another test available for HCV is an immunoblot assay (RIBA-2). Both methods are of limited use, however, because a period of several weeks separates infection and seroconversion. Recently, a genetic polymorphism near the IL28B gene, encoding IFN-lambda-3, has been reported to be associated with an approximately twofold change in response to treatment [12].

Biomarkers of Nonalcoholic Fatty Liver Disease

Serum proteomics are of importance for biomarker research in nonalcoholic fatty liver disease (NAFLD) but have not been implemented in clinical practice [13]. Recently, 4-hydroxynonenal-protein adducts have been suggested as a reliable biomarker of lipid oxidation in liver diseases [14].

Biomarkers of Liver Fibrosis and Cirrhosis

The current “gold standard” for liver cirrhosis detection is an invasive, costly, often painful liver biopsy. Therefore, there is a need for biomarkers that could obviate biopsy in cirrhosis patients. Among the noninvasive alternatives to liver biopsy, several studies have demonstrated the predictive value and a better benefit-to-risk ratio than biopsy of five combinations of simple serum biochemical markers i.e., FibroMAX in patients at risk of chronic liver diseases such as patients with chronic hepatitis B or C: FibroTest for the quantitative assessment of fibrosis in patients with chronic hepatitis or drug-induced liver damage; SteatoTest for the quantitative assessment of steatosis in fatty liver disease; ActiTest for the quantitative

assessment of necroinflammatory activity in chronic viral hepatitis C and B and Nash Test for the categorical diagnosis of nonalcoholic steatohepatitis; and AshTest for the quantitative assessment of alcoholic steatohepatitis (known in the USA as HCV FibroSURE, HBV FibroSURE, ASH FibroSURE, and NASH FibroSURE) [15].

Emerging Biomarkers in Chronic Liver Diseases

Activin

Activin is a cytokine, which belongs to the transforming growth factor-β superfamily and is released rapidly into the circulation during inflammation [16, 17]. Studies suggest an involvement of activin A not only in fibrosis but also in lipid accumulation [16]. Therefore it is used as a diagnostic marker of clinical inflammation and probably plays a therapeutic role [17].

Follistatin

Follistatin is an activin-binding protein. Although elevated follistatin can be detected both in tumor tissue and in the peripheral blood, it has no benefit as surveillance biomarker for HCC development in patients with alcoholic and nonalcoholic liver disease because of its already elevated levels in the underlying liver pathologies [16].

Thymosin β4 (T β4)

Elevated serum concentrations) of T β4 might be a defense mechanism counteracting ongoing inflammation and fibrogenesis [18, 19].

C-reactive Protein

CRP is an acute-phase protein produced mainly by hepatocytes. It is produced as an acute-phase reactant predominantly in the liver, in response to a variety of acute and chronic inflammatory conditions, and is an important component of the innate immune system [1, 4, 6]. The hepatic inflammatory response of CRP to injury and the production of proinflammatory cytokines are the main factors driving stellate

cell activation and fibrogenesis [20]. Serum high-sensitivity CRP (hs-CRP) is measured by immunoturbidimetry or by multiplex enzyme-linked immunosorbent assay (ELISA)-based assays [20, 21].

IL-6

Interleukin-6 (IL-6) is a proinflammatory cytokine derived from the adipose tissue. IL-6 induces secretion of CRP in the liver and, hence, may contribute to hepatocarcinogenesis [22].

TNF-α

Tumor necrosis factor-alpha (TNF-α) is a proinflammatory cytokine, and emerging data leads to the conclusion that serum TNF-α levels could be used as a sensitive predictor of liver inflammation and even hepatic malfunctions [23, 24].

Adiponectin

Adiponectin is a protein, secreted from adipose tissue. High concentrations of adiponectin were associated with higher risk of hepatocellular carcinoma [25].

All these biomarkers are not used in the routine clinical practice but are expected to be integrated in future algorithms of diagnosing and treating patients with cirrhosis.

Biomarkers of Chronic Inflammatory Conditions in Neurology

Regarding biomarkers of chronic inflammatory conditions in neurology, most studies have concentrated on the discovery, characterization, and validation of several highly promising individual biomarkers, but their impact on different disease stages has hardly been extensively investigated. One of the primary goals of future studies on biomarkers should therefore be the evaluation and validation of given markers according to their impact on diagnosis of subjects at risk, differential diagnosis at early clinical stages, and predication and description of disease course. Future research should also focus on the development and validation of cost-effective and broadly available high-throughput technologies for biomarker quantification, as this

seems the only way to address the need for highly accurate diagnosis and sufficient supervision of therapeutic strategies.

Biomarker Development in Chronic Inflammatory Demyelination

Biomarkers in Central Nervous System Chronic Inflammation

Multiple sclerosis (MS) is the most important chronic inflammatory disease of the central nervous system (CNS) with a complex pathophysiological course that includes inflammation, demyelination, axonal damage, and repairing [26]. MS is characterized by heterogenous genetic backgrounds and immunopathogenetic subtypes, which are reflected in variable clinical disease courses and unpredictable therapeutic effects. Therefore it seems more credible that a panel of different biological markers, rather than a single antibody or other biological marker, should have to be discovered to reflect the various stages of inflammation, demyelination, axonal degeneration, and remyelination [27].

Despite the progress in new technologies (such as DNA microarrays, real-time polymerase chain reaction (PCR), multicolor flow cytometry) and the advances in the knowledge of the MS pathogenesis, the body of scientific evidence obtained so far cannot support the existence of reliable biological markers. Most of the peripheral blood markers under consideration are of little reproducibility, while biological markers in the cerebrospinal fluid (CSF) are of little utility due to inability of repeated sampling [28]. Biomarkers of disease activity in MS could help in predicting the disease course and treatment response, thus providing relapse monitoring, treatment guidance, and long-term outcome improvement.

Unseparated blood is used both for flow cytometry analysis of cellular subpopulations and for PCR studies. After coagulation, serum can be used for the measurement of soluble markers, such as antibodies and cytokines, while after separation procedures of uncoagulated samples, different cellular populations can be used for functional studies [29]. Numerous cytokines (TNFa, IL-12, IL-17, IL-23, INF-γ), cell surface markers, adhesion and migration markers, antibodies, and other markers of tissue damage have been explored as serum or CSF biomarkers for disease activity, but none of them has so far the necessary validated reliability for widespread clinical use [30, 31]. This could probably be attributed to the fact that MS is not a systemic disease and therefore serum molecules might be not able to depict the immunological changes that take place in the CNS.

Autoantibodies against myelin (anti-MOG, anti-MBP, anti-PLP, anti-GAGA4) have been thoroughly studied, but none of them has so far demonstrated convincingly a MS-specific antibody response to a certain CNS target antigen. Moreover, most of the antibodies, detected in MS, are also found in other disease conditions and, to a lower extent, in healthy controls. Among the aforementioned antibodies,

myelin oligodendrocyte glycoprotein (MOG) appears to be the most promising marker. MOG was initially identified as a dominant target antigen for demyelinating antibodies in experimental autoimmune encephalomyelitis, and its role in the pathogenesis of MS still remains unresolved and controversial [32].

To date, 24S-hydroxycholesterol is the only promising biomarker related to neuronal damage in peripheral blood. 24S-hydroxycholesterol is a cholesterol metabolite specific to the brain, formed by the catalytic activity of the cytochrome P450 enzyme, and thus could serve as a marker for changes in brain cholesterol turnover caused by demyelination or neurodegeneration [33]. A moderate correlation was found between the numbers of apolipoprotein e4 (ApoE4) alleles and MS disease progression, while the concentration of serum ApoE was not found to be consistently abnormal in all groups of MS patients. Apart from lipid transport and cholesterol homoeostasis, evidence suggests that ApoE is also involved in the blood–brain barrier maintenance and in oxidative stress protection [33].

Serum fluctuations of β2 microglobulin (β2-MG), the 12 kDa light chain of the class I major histocompatibility complex (MHC-I) on the surface of many cells, and neopterin, a low molecular mass molecule that is synthesized from guanosine triphosphate, have been found to be good indicators of treatment effect but not of disease activity in MS. During the natural course of MS, β2-MG was found to be stable over time, and although urinary excretion of neopterin was found to be higher during clinical relapses, neopterin blood levels were not found to correlate with clinical and MRI measurements. Furthermore, both neopterin and β2-MG are unstable and rapidly eliminated by the kidneys, and thus these molecules can be easily detected in urine specimens [34]. Urine collection is a simple and noninvasive process; however, disabled multiple sclerosis patients often have chronic urinary tract infections or asymptomatic bacterial bladder colonization—due to bladder instability—which both can negatively affect the urine biomarker results. Currently, measurable urine biomarkers include neopterin, nitrate/nitrite, prostaglandin metabolites, b2-MG, immunoglobulin (Ig) light chains, interleukins (IL-1, IL-2, sIL-2R, IL-6, IL-8), and myelin basic protein (MBP)-like material [29].

Finally, possible biomarkers in cerebrospinal fluid (CSF) that reflect key pathological processes of MS in inflammation, immune Th1 dysfunction, demyelination, oxidative stress, remyelination, and neuroaxonal damage are being investigated [35]. Currently, the main role of CSF examination in MS is to support the diagnosis of MS and to exclude other diagnoses. The most useful markers are currently the presence of two or more IgG oligoclonal bands (OCBs), as well as an elevated IgG index. However, numerous other CSF biomarkers are under investigation, including kappa free light chains (KFLC), anti-myelin antibodies, sVCAM-1 (soluble vascular cell adhesion molecule-1), and 24S-hydroxycholestrol [36]. At present none of them, except for the OCBs, fulfills the criteria of applicability in clinical practice and should therefore be further investigated and validated in future studies [37]. As for CSF oligoclonal banding test, it is estimated to have sensitivities between 69 and 91% with specificities between 59 and 94% for the diagnosis of MS, and when combined with MRI studies, both sensitivity (56–100%) and specificity (53–96%) are enhanced [38]. Other biomarkers that are currently used in clinical practice in the diagnosis or treatment of MS are mentioned in Table 3.

Table 3 Biomarker development in chronic inflammatory central nervous system demyelination

Biomarker	Biomarker abbreviation	Origin	Technique	Prevalence	Remarks
IgG oligoclonal bands	OCBs	Serum, CSF	Isoelectric focusing combined with immunoblotting	>95% of MS patients	Can be found in patients with demyelinating disease
IgG index	–	Serum, CSF	(CSF/serum IgG)/(CSF/serum albumin	Increased in 70% of MS patients	Can be found in patients with demyelinating disease
Anti-aquaporin-4 antibodies	*anti*-AQP4	Serum, CSF	Various assays	Almost absent in MS patients	75–90% of patients with neuromyelitis optica
Neutralizing antibodies	Nab	Serum	Various assays	2–45% of patients treated with INFb	If present, a switch to a non-INFb treatment should be considered
Anti-natalizumab antibodies	–	Serum	ELISA	4.1–6.1% of patients treated with natalizumab	If persistently positive, treatment with natalizumab should be discontinued
Anti-JC virus antibodies	Anti-JVC ab	Serum	ELISA	50–60% of MS patients	Estimation of the patient's risk for PML if treated with natalizumab
Anti-VZV antibodies	Anti-VZV ab	Serum	ELISA	90–95% of MS patients	Seronegative patients should be vaccinated at least 1 month before start of fingolimod

CSF cerebrospinal fluid, *MS* multiple sclerosis, *INFb* interferon b, *ELISA* enzyme-linked immunosorbent assay, *JC* John Cunningham, *PML* progressive multifocal leukoencephalopathy, *VZV* varicella-zoster virus

Biomarkers in Peripheral Nervous System Chronic Inflammation

Chronic inflammatory demyelinating polyneuropathy (CIDP) is an autoimmune demyelinating disease of the peripheral nervous system. Due to the high clinical heterogeneity and lack of a specific confirmatory biomarker, at least 14 different sets of diagnostic criteria (with varying sensitivities) have been proposed so far. Ancillary diagnostic tests include the measurement of cerebrospinal fluid protein levels, nerve biopsy, electrodiagnostic testing, and treatment response [39, 40].

High titers of IgM antineural antibodies to myelin-associated glycoprotein (MAG), sulfatide, and gangliosides (GM1, GM2, GD1a, GD1b) are highly predictive of a chronic immune-mediated neuropathy; however, they are not specific and thus cannot be strictly associated with a definite clinical syndrome [41]. Transthyretin (TTR)—a haptoglobin isoform—was highlighted in a small pilot study of CSF proteome analysis in patients with CIDP as a promising marker that warrants further evaluation [42].

The heterogeneity in therapeutic responses to immunoglobulin (IVIg), steroids, or plasmapheresis necessitates the need for identifying biomarkers to determine the most suitable therapy and to monitor the therapeutic response in patients with CIDP [43]. Potential biomarkers that may help to guide therapy and treatment response with IVIG include the alterations in transient axonal glycoprotein-1 (TAG-1) and measurement of Fcγ RIIB density on B cells, following infusion of IVIG [40].

Biomarkers of Chronic Inflammatory Diseases of the Skin

Scleroderma

Skin involvement is of major prognostic value in systemic sclerosis (SSc) and often the primary outcome in clinical trials. Nevertheless, an objective, validated biomarker of skin fibrosis is lacking. Optical coherence tomography (OCT) is an imaging technology providing high-contrast images with 4 μm resolution, comparable with microscopy (“virtual biopsy”) [44]. It has been also suggested that levels of adiponectin, a marker for PPAR-gamma activity, correlate with skin fibrosis in systemic sclerosis [45].

Dermatitis Herpetiformis

The deamidated gliadin peptides (DGP) cross-linked to human tissue transglutaminase (tTg) comprise a novel neo-epitope structure (Neo-tTg) for serological screening of celiac disease (CD). Neo-epitope tissue transglutaminase autoantibodies have been suggested as a useful biomarker of the gluten-sensitive skin disease called dermatitis herpetiformis [46].

Psoriasis

Oxidative stress was implicated in the psoriasis disease development and may damage DNA leading to keratinocytes cell death. According to an interesting study, serum 8-OHdG levels could be used as good biomarker for the early diagnosis of psoriasis and its management [47]. Other serum biomarkers for psoriasis are YKL-40 (chitinase 3-like-1), which has been suggested as a biomarker for psoriasis vulgaris and pustular psoriasis, and beta-defensin-2 protein as a serum biomarker for disease activity in psoriasis which reaches biologically relevant concentrations in lesional skin [48].

Of interest, plasma levels of transforming growth factor (TGF)-beta1, tissue inhibitors of metalloproteinases (TIMP)-1, matrix metalloproteinase (MMP)-1, and interleukin(IL)-18, when analyzed separately, demonstrate an association with psoriasis severity and treatment efficacy [49]. Finally, soluble tumor necrosis factor-alpha receptor type 1 has been suggested as a biomarker of response to phototherapy in patients with psoriasis [50].

Atopic Dermatitis

Several cytokines/chemokines, especially in breast milk, are potential biomarkers for development of atopic dermatitis in early infancy [51]. Recently, urinary eosinophil protein X has been suggested as a novel inflammatory biomarker that identifies children at risk of developing atopic disease [52].

Graft-Versus-Host Disease of the Skin

Graft-versus-host disease (GVHD), the major complication of allogeneic bone marrow transplantation, affects the skin, liver, and gastrointestinal tract. There are no plasma biomarkers specific for any acute GVHD target organ [53]. Recently serum elafin has been suggested as a valuable biomarker of graft-versus-host disease of the skin [54].

Dermatomyositis

In patients with polymyositis and dermatomyositis, KL-6, which is a mucin-like high-molecular-weight glycoprotein, has been suggested as a promising biomarker for use in clinical practice to assess clinical response to treatment [55].

Biomarkers of Chronic Autoimmune Rheumatic Diseases

Autoimmune rheumatic diseases (ARDs) comprise a wide variety of chronic inflammatory disorders in which innate and adaptive immune responses lead to autoimmune-mediated tissue damage. While the etiology of ARDs remains unclear, multiple genetic, epigenetic, hormonal, and environmental influences appear to play a role in disease pathogenesis. The spectrum of their clinical manifestations is characterized by great diversity in terms of disease severity and the extent of organ involvement. Most ARDs run a relapsing-remitting course, yet the pattern of continuously active disease is described in a considerable proportion of patients diagnosed with these chronic inflammatory illnesses. Overall, ARDs affect approximately 5% of the population and result in substantial morbidity and increased mortality. Furthermore, these diseases place a significant burden on public health with high financial costs [56, 57].

The identification of biomarkers that measure the underlying biologic processes in ARDs reliably and reproducibly has been recognized as a growing need in rheumatology, but given the complex pathogenesis of these disorders, heterogeneous clinical manifestations, and varying rates of disease progression among patients, it is reasonable to assume that a particular biomarker may reflect only one specific aspect, rather than all aspects of the disease course at any given time. Therefore, some biomarkers provide prognostic information regarding severity of the disease, whereas others predict response to therapy optimizing risk/benefit assessment in individual patients. Likewise, biomarkers might predict or quantify the risk of ARDs in individuals or populations, and others could establish or confirm the diagnosis of a specific ARD.

This section will only focus on biomarkers detected through blood tests that have established clinical utility in major ARDs.

Rheumatoid Arthritis

Rheumatoid arthritis (RA) is an ARD that primarily affects the joints and is associated with progressive functional disability, systemic complications, high socioeconomic costs, and a guarded prognosis. While the exact prevalence across the entire population is unknown, available data suggest that it affects about 1% of the population, making it one of the most common ARDs [58]. RA involves a complex interaction among genotype and environmental triggers, leading to a breakdown of immune tolerance with autoantibody production and synovial inflammation in a characteristic symmetric pattern. Distinct mechanisms regulate inflammation and matrix destruction, including damage to bone and cartilage [59].

The diagnosis of RA remains clinical and is based on several criteria, including physical symptoms, joint radiographs, and serological tests [60]. Recently, emerging data suggest that a preclinical period precedes the onset of clinically apparent

RA, which is characterized by the presence of abnormalities in disease-related biomarkers. During this period many genetic and environmental risk factors are likely to act to initiate or propagate autoimmunity [61].

The main clinically useful biomarkers in patients with RA are rheumatoid factors (RF) and antibodies to citrullinated peptides (ACPA) for diagnosis and prediction of functional and radiographic outcomes. These autoantibodies, when appearing during the preclinical period, may predict the disease onset [61]. Other useful laboratory biomarkers are erythrocyte sedimentation rate (ESR) and C-reactive protein (CRP).

Genetic Biomarkers in RA

Susceptibility to RA is clearly defined by a pattern of inherited genes, with the human leukocyte antigen (*HLA*)-DRB1 locus being the most important biomarker, particularly in patients who are RF or ACPA positive. However, other risk alleles that consistently aggregate functionally with immune regulation, including cytokine promoters and genes involved in T-cell stimulation, activation, and functional differentiation, contribute to susceptibility and disease severity [59].

Rheumatoid Factors

RF is an antibody directed against the constant (Fc) portion of IgG. The key pathogenic markers in RA are IgM and IgA RFs. They are found in 75–80% of RA patients (Table 4) at some time during the disease course. High titer IgM RF is relatively specific for the diagnosis of RA in the context of a chronic symmetric polyarthritis and was, for decades, the only serologic criterion widely used in the diagnosis of RA. Nevertheless, RFs may also be present in other ARDs and chronic infections (Table 4). RFs in RA patients are distinguished from RFs in healthy individuals in that they exhibit affinity maturation [62]. RF has little predictive value in the general population, because the disease prevalence is relatively low.

RF may have some prognostic value with regard to disease manifestations and activity, as well as the severity of joint erosions. Seropositive RA (i.e., disease associated with a positive RF test) is often correlated with more aggressive joint disease and is more commonly complicated by extra-articular manifestations than seronegative RA [62]. Rheumatoid nodules and vasculitis occur almost exclusively in seropositive patients. A positive RF test at the initial evaluation is associated with more destructive joint pathology. It has also been proposed that the rate of formation of new erosions among seropositive patients correlates with the RF titer [62]. The presence of RF increases the likelihood of a clinically significant response to rituximab which is a monoclonal antibody against the CD20 antigen found on the surface of B lymphocytes after failure of tumor necrosis factor (TNF)-alpha inhibitor therapy. Finally, the presence of RF, particularly at high titers, may antedate the clinical development of RA [61, 63].

Table 4 Biomarkers in major autoimmune rheumatic diseases

Biomarker	Biomarker abbreviation	Origin	Technique	Sensitivity/specificity	Remarks
Rheumatoid factor	RF	Serum	Agglutination of IgG-sensitized sheep red cells or of bentonite or latex particles coated with human IgG; radioimmunoassay; enzyme-linked immunosorbent assay; nephelometry	75–80%/85% (for rheumatoid arthritis)	Can be found positive before rheumatoid arthritis diagnosis
Anti-citrullinated peptide antibodies	ACPA	Serum	Enzyme-linked immunosorbent assay (ELISA)	50–75%/95–97% (for rheumatoid arthritis)	Can be found positive before rheumatoid arthritis diagnosis
Antinuclear antibody	ANA	Serum	Indirect immunofluorescence assay; enzyme-linked immunosorbent assay (ELISA)	98% (for systemic lupus erythematosus)/may be positive in other rheumatic disease, organ-specific autoimmune diseases, chronic infections, and lymphoproliferative diseases	Can be found positive before systemic lupus erythematosus diagnosis
Anti-double-stranded DNA antibody	Anti-dsDNA	Serum	Farr radioimmunoassay; *Crithidia* immunofluorescence assay; enzyme-linked immunosorbent assay (ELISA)	70%/95% (for systemic lupus erythematosus)	Can be found positive before systemic lupus erythematosus diagnosis
Anti-Smith antibody	Anti-Sm	Serum	Immunodiffusion assay; enzyme-linked immunosorbent assay (ELISA)	20–30%/99% (for systemic lupus erythematosus)	Can be found positive before systemic lupus erythematosus diagnosis

(continued)

Table 4 (continued)

Biomarker	Biomarker abbreviation	Origin	Technique	Sensitivity/specificity	Remarks
Anti-Ro/SSA	Anti-Ro/SSA	Serum	Enzyme-linked immunosorbent assay (ELISA); immunoblot assay; immunodiffusion assay	30% (for systemic lupus erythematosus)/may be positive in Sjogren's syndrome	Can be found positive before systemic lupus erythematosus diagnosis
Anti-La/SSB	Anti-La/SSB	Serum	Enzyme-linked immunosorbent assay (ELISA); immunoblot assay; immunodiffusion assay	15–20% (for systemic lupus erythematosus)/may be positive in Sjogren's syndrome	Can be found positive before systemic lupus erythematosus diagnosis
Anti-ribonucleoprotein antibody	Anti-RNP	Serum	Immunodiffusion assay; enzyme-linked immunosorbent assay (ELISA)	30% (for systemic lupus erythematosus)/may be positive in mixed connective tissue disease and scleroderma	Can be found positive before systemic lupus erythematosus diagnosis
Anti-neutrophil cytoplasmic antibodies	C-ANCA	Serum	Immunofluorescence assay; enzyme-linked immunosorbent assay (ELISA) for proteinase-3 (PR3)	60–90% (for Wegener granulomatosis)/both a C-ANCA pattern on immunofluorescence and PR3-ANCA positive by ELISA are highly specific for Wegener granulomatosis	C-ANCA gives positive immunofluorescence result with cytoplasmic staining pattern
Anti-neutrophil cytoplasmic antibodies	P-ANCA	Serum	Immunofluorescence assay; enzyme-linked immunosorbent assay (ELISA) for myeloperoxidase (MPO)	70% (for microscopic polyangiitis)/both a P-ANCA pattern on immunofluorescence and MPO-ANCA positive by ELISA are highly specific for ANCA-associated vasculitis mostly microscopic polyangiitis	P-ANCA gives positive immunofluorescence result with perinuclear staining pattern

Anti-citrullinated Peptide Antibodies

ACPA are directed against citrullinated protein epitopes and are detected by use of ELISA for antibodies against synthetic cyclic citrullinated peptides. The sensitivity of ACPA assays for RA varies from about 50 to 75%, depending upon the assay and study population, while specificity of ACPA for RA is relatively high, usually >90% (Table 4). ACPA-positive patients with early RA are at increased risk of progressive joint damage, while ACPA testing may predict erosive disease more effectively than RF. The presence of ACPA is also predictive of more rapid radiographic progression [62]. Positive ACPA testing also appears to predict an increased risk for radiographic progression in patients with early oligo- or polyarthritis who are IgM RF negative. A decrease in ACPA titers can be seen in patients treated effectively, particularly if treated early with nonbiologic or biologic disease-modifying antirheumatic drugs (DMARDs), but is less frequent and of a lesser magnitude than the decrease in IgM RF. ACPA may be detectable months or even years before the development of RA, and the proportion of individuals who are seropositive for ACPA increases progressively until clinical onset of disease [61, 63]. Finally, this biomarker is useful in the differential diagnosis of early polyarthritis, because of the relatively high specificity for RA of these autoantibodies. The presence of ACPA is highly predictive of future development of RA with positive predictive values of >90% in most studies [61, 63].

Erythrocyte Sedimentation Rate

The rate at which erythrocytes fall through plasma, the ESR, depends largely upon the plasma concentration of fibrinogen and can be influenced by the size, shape, and number of red cells, as well as by other plasma constituents. Thus, results may be imprecise and sometimes misleading. Despite these shortcomings, an elevated ESR in patients with early RA is predictive of greater radiographic joint damage in subsequent years despite treatment with conventional DMARDs. ESR values tend to correlate with disease activity in RA as well as disease severity and may be useful for monitoring therapeutic response.

C-reactive Protein

CRP has been recommended as an objective measure of disease activity in RA. Unlike the ESR, CRP can be measured using stored serum samples; it is independent of the hemoglobin concentration and can be performed in automated serum analyzers. Radiographic damage is significantly more likely to progress when CRP and ESR are elevated, irrespective of the presence or absence of RF and irrespective of therapeutic intervention. Elevations of both ESR and CRP are stronger indications of radiologic progression than CRP alone. However, a wide variation in the relationship between the degree of radiographic change and cumulative CRP was noted in several studies between patients, particularly those with low CRP levels.

Other Investigational Biomarkers in RA

Cytokines are small proteins that regulate the immune system and participate in intercellular communications. There is more information on the role of cytokines in RA than in any other ARD which has led to clinical trials with several novel agents designed to interrupt the cytokine network of this disease. Anti-cytokine therapy has shown clear evidence of clinical efficacy, especially with TNF-alpha-directed therapeutic approaches. Overexpression of certain cytokines, such as Il-2, Il-6, IL-8, IL-17, IL-21, TNF-α and granulocyte-macrophage colony-stimulating factor have been reported in RA. However, discrepancy in the results of several studies, differential cytokine levels in sera and synovial fluid, analytical variability among immunoassays, and certain limitations of cytokine measurements [64] preclude their use as reliable biomarkers in everyday clinical practice.

Systemic Lupus Erythematosus

Systemic lupus erythematosus (SLE) is the most heterogeneous ARD with more than 100 autoantibodies found in patients and a disease spectrum ranging from mild symptoms to life-threatening multi-organ manifestations. The hallmark characteristics of SLE, including production of autoantibodies, deposition of immune complexes in tissues, and excessive complement activation, are generally thought to be consequences of immune dysregulation. Owing to its complex etiology and pathogenesis, diverse clinical presentation, and unpredictable course, SLE remains one of the greatest challenges in the field of rheumatology [65].

Genetic Biomarkers in SLE

The genome-wide association study (GWAS) approach has accelerated the discovery of genetic variations contributing to SLE. Over 50 loci associated with SLE susceptibility have been identified, and most encode gene products participating in the key pathways relevant to SLE pathogenesis, including those encoding the early components of the complement system, cytokines, chemokines, loci that mediate signaling transduction in B and T cells, as well as variants implicated in immune complex clearance. Yet, dysregulation of type I interferon (IFN) appears as a central driver of SLE pathogenesis [66, 67]. Variants of *FCGR2A* also represent significant risk factors for the disease, whereas the FCG3A-V/F158 polymorphism has a substantial impact on the development of lupus nephritis [68, 69].

Biomarkers for Preclinical Disease Stage and for SLE Diagnosis

Individuals who develop SLE appear to have an initial preclinical stage of "benign autoimmunity" which is characterized by antinuclear antibody (ANA) positivity and detection of several autoantibodies (anti-Ro/SSA, anti-La/SSB, anti-phospholipid). This phase develops into a more aggressive stage of "pathogenic autoimmunity" characterized by the presence of antibodies targeting double-stranded DNA (dsDNA), the Smith (Sm) antigen, and ribonucleoproteins (RNP) that in turn rapidly evolves into clinically apparent disease and tissue inflammation [63]. While certain biomarkers such as anti-dsDNA and anti-Sm are elevated relatively shortly before SLE onset, the relationship with preclinical autoimmunity remains uncertain for ANAs which have been shown to be present at titers of >1/40 in up to 27% of subjects, most of whom will never develop SLE or other ARD [63].

Although the American College of Rheumatology (ACR) developed classification criteria for SLE (published in 1982 and revised in 1997), these criteria are often cited to support a lupus diagnosis; yet this approach is problematic in clinical practice. Traditionally, determination of autoantibodies (Table 4) such as ANA, anti-extractable nuclear antigen antibodies (anti-Ro/SSA, anti-La/SSB, anti-RNP, and anti-Sm), and anti-dsDNA is used in diagnosing and monitoring SLE. ANAs are present in virtually all SLE patients, whereas anti-dsDNA and anti-Sm are highly specific for the disease (Table 4). Nevertheless, there are considerable drawbacks to the use of these immunologic markers [65]. Recently, cell-bound complement activation products have been proposed as more sensitive biomarkers for SLE diagnosis compared to other autoantibodies [67], but larger studies are needed to provide a more definite confirmation of their validity.

Biomarkers for SLE Disease Activity

Currently, disease activity in SLE is frequently assessed using composite indices which include a variety of clinical and laboratory parameters. Laboratory measures of complement and autoantibodies are components of most disease indices. Several studies have been conducted to identify the associations of various autoantibodies, particularly anti-dsDNA and complement proteins (including C3 and C4 as well as activation products) with SLE disease activity. The results, however, are inconsistent, and such uncertainty may also confound the assessment of disease activity with the widely used disease indices. Therefore, the value of conventional tests measuring serum complement and autoantibodies, as biomarkers for SLE disease activity, is being revisited. Several candidate biomarkers have emerged recently; alteration of the B cell subpopulation (namely, the increase in $CD27^{high}$ plasma cells), cell-bound complement activation products, IFN-inducible genes expression and/or serum levels of IFN-inducible chemokines, serum levels of B lymphocyte stimulator, cytokines (IL-6, IL-10, IL-16, and IL-18), and anti-nucleosome antibodies may be valuable biomarkers for monitoring disease activity [67].

Lupus Biomarkers for Specific Organ Involvement

SLE can affect practically any tissue and organ. Nevertheless, not all organs are affected simultaneously, and involvement of a specific organ will not necessarily be manifested in the same manner in all patients. Lupus patient care would benefit immensely from biomarkers that could determine and/or predict organ-specific disease.

Among the numerous manifestations of SLE, renal involvement is one of the most common, and it continues to cause significant morbidity and mortality. Laboratory biomarkers such as creatinine clearance, proteinuria, urine sediment, serum C3 and C4, and anti-dsDNA have for decades been used to follow the onset, course, and severity of lupus nephritis [65], yet, it is generally recognized that these measurements are inadequate. Current efforts are focused on identification of more sensitive and specific biomarkers to diagnose and monitor renal disease in SLE. Anti-nucleosome antibodies may be more sensitive and offer better diagnostic performance than anti-dsDNA for active disease, especially nephritis, in SLE patients. Other recent studies demonstrate a strong correlation between the presence of anti-C1q antibodies and lupus nephritis and suggest that anti-C1q determination may serve as a biomarker to monitor renal involvement and/or predict renal flares. Other promising biomarkers include the serum neutrophil gelatinase-associated lipocalin which might predict renal relapses, yet longitudinal studies of adult lupus are needed [67].

Many patients with SLE experience a wide range of neuropsychiatric (NP) manifestations that result predominantly from immune-mediated damage of the central nervous system (CNS). While NPSLE is a common manifestation, its diagnosis is extremely difficult due to lack of reliable biomarkers. Because autoantibodies are clearly involved in tissue damage in other organs, autoantibodies reactive to CNS antigens naturally become the focus of the investigation of NPSLE pathogenesis and the pursuit for reliable biomarkers. Stroke in patients with SLE is likely to reflect thrombosis due to antiphospholipid antibodies. In addition, recent studies provide convincing evidence that a subset of anti-dsDNA antibodies cross-react with a pentapeptide consensus sequence that is present in the extracellular domain of the *N*-methyl-D-aspartate (NMDA) receptor. NMDA receptors bind the neurotransmitter glutamate expressed by neurons throughout the forebrain and particularly at the hippocampus. If such cross-reacting autoantibodies enter the brain upon transient breach of the blood–brain barrier, they may bind to antigens expressed in different regions of the brain and induce non-inflammatory neuronal injury resulting in various neurologic and psychological changes [67]. Antibodies to ribosomal P proteins (anti-P) were initially considered extremely promising biomarkers for the diagnosis of lupus psychosis and of mood disorders. Yet, it was demonstrated that anti-P antibody testing has negligible diagnostic utility for NPSLE overall or for these particular manifestations [70]. Testing for anti-P antibody is not useful in excluding disease-mediated psychosis or mood disorder with enough certainty, since more than 60% of cases are false negative. Also, a false-positive rate of ~20% militates against the dependence on this laboratory test for diagnosing psychiatric disorders in lupus patients [70].

Vasculitis

Vasculitis comprises a heterogeneous group of ARDs that is characterized by inflammatory destruction of blood vessels which become liable to occlude or rupture. Depending on the size, distribution, and severity of the affected vessels, vasculitis can result in clinical syndromes that vary in severity from a minor self-limited rash to a life-threatening multisystem disorder.

The most important primary systemic vasculitis syndromes are granulomatosis with polyangiitis (GPA, formerly known as Wegener's granulomatosis) and microscopic polyangiitis (MPA) which are associated with circulating autoantibodies to neutrophil cytoplasmic antigens (ANCA). It has been suggested that they be grouped together as ANCA-associated vasculitis because of their histologic similarities, the absence of immune deposits in involved tissues, the potential contribution of ANCA to their pathogenesis, and their similar responses to immunosuppressive therapy. Renal involvement occurs in 70% of affected patients and is manifested as rapidly progressive glomerulonephritis; it results in either death or end-stage renal failure within 2 years in more than 40% of patients. MPA and GPA were once considered life-threatening ARDs, but immunosuppressive therapy has substantially improved the survival of affected patients [71, 72].

Biomarkers for GPA and MPA

The most useful biomarkers for diagnosis of ANCA-associated vasculitis include antibodies to proteinase-3 (PR3) or myeloperoxidase (MPO) which are highly specific for GPA and MPA, respectively (Table 4). Inflammatory markers such as ESR and CRP exhibit only modest sensitivity for active early vasculitis in untreated patients [71, 72].

All of these biomarkers are problematic for use in assessing disease activity in patients with established diagnoses. Many relapses of GPA or MPA are accompanied by increases in anti-PR3 or anti-MPO titers; yet these markers do not appear to have the same predictive value that they do before treatment, making their use controversial.

Laboratory biomarkers such as creatinine clearance, proteinuria, and urine sediment are thought to possess very high sensitivity for active glomerulonephritis, that is, a normal urinalysis is regarded as ruling out glomerulonephritis. Similarly, the presence of red blood cell casts is regarded as having high specificity, albeit low sensitivity for glomerulonephritis. Nevertheless, once renal damage has occurred due to glomerulonephritis, proteinuria, hematuria, and even red blood cell, casts may persist without evidence of progressive kidney disease.

Among many markers of inflammation, angiogenesis, tissue damage, and repair that were measured in GPA and MPA patients, the most promising include MMP-3, tissue inhibitor of metalloproteinase-1, and CXCL13 (B-cell attracting chemokine 1) [71, 72].

Novel Technologies for Biomarker Development

Metabolomics is a novel and powerful technique for studying biological systems that allows for the analysis of the specific response of organisms to environmental stimuli. This new approach belongs, along with genomics, proteomics, and transcriptomics, to the family of "-omics" sciences. It is based on non-hypothesis-driven scanning platforms for identifying biomarkers and profiling the patients. Thus, the fundamental rationale in metabolomics is that perturbations caused by a disease in a biological system will lead to correlated changes in concentrations of certain metabolites. This procedure has become feasible only recently with the advent of new high-throughput technologies, including mass spectrometry and nuclear magnetic resonance. Comparison of patients with active RA and those in remission provided different baseline metabolic profiles, suggesting that efficacious treatment may affect biological changes for improved metabolic profiles. In SLE, the disease influences the metabolite profile and particularly energy, amino acid, lipid, and purine metabolism [73].

Proteomic approaches include gel-based methods such as two-dimensional difference gel electrophoresis (2D DIGE) and modern mass spectrometric techniques to identify encoded proteins that may better reflect cell function and disease. Most profiling studies in ARDs have been conducted in RA. Using high-throughput mass spectrometric techniques, these studies have uncovered about 33 different proteins that are differentially expressed in RA, of which 3 (serum amyloid A, superoxide dismutase, and triose phosphate isomerase) are of particular interest as their elevations in plasma have been reported in multiple studies. Proteomics can also yield insights into the molecular pathways impacted by therapy. A recent example is the identification of the nuclear factor-kappa B pathway as being differentially expressed in RA patients treated with anti-TNF-alpha agents [74].

Transcriptomic profiling using DNA microarrays has been applied to the study of several ARDs. The analysis of antibody repertoires with antigen microarrays found that those RA patients who had antibodies to citrullinated peptides in which peptidylarginine has been converted to peptidylcitrulline were more prone to develop severe disease. In another SLE study, the investigators developed antigen microarrays including several SLE-related antigens, and they found clusters of antibody reactivity associated to glomerulonephritis and overall disease activity [75–77].

Biomarker discovery and development is a rapidly growing field in rheumatology, which is nevertheless fraught with several limitations. Important amendments to the translational research enterprise are necessary to address these challenges. Hence, imperative alterations should include rigorous design for studies of biomarkers that would allow valid interpretation of their clinical utility. In particular, for studies of biomarkers for diagnosis, greater attention to potential confounders would help ensure accuracy. When examining biomarkers of disease activity, emphasis should be placed on evaluating these markers longitudinally. In research regarding biomarkers of prognosis, longitudinal design and the use of validated outcome measures would provide reliable results in order to enable use in clinical

decision-making process of novel biomarkers that outperforms currently available tests. Finally, the development of novel high-throughput assays might hold tremendous potential for shaping how ARDs are diagnosed, prognosticated, and managed clinically over the coming years.

Future Trends

Personalized medicine is the best way to integrate new biotechnologies into medicine for improving the understanding of pathogenesis of diseases and management of patients. Development of personalized medicine is closely linked to biomarkers, which may serve as the basis for diagnosis, drug discovery, and monitoring of diseases.

Biomarker discovery is an ongoing process, with translation being tested de novo in every single study, providing us with the opportunity to revise our knowledge of the complex scheme of human physiology and pathophysiology.

An ideal biomarker must be specifically associated with a particular disease or disease state and be able to differentiate between similar physiological conditions. A rapid, simple, accurate, and inexpensive detection of the relevant marker should be available, together with a measurable and standard baseline as a reference point.

In the active search for new biomarkers, many potential candidates can be considered side by side, allowing many failures but a few winners. The traditional identification of biomarkers as an observational side product of clinical practice is increasingly turning into an industrialized process of biomarker discovery, supported by standardized paradigms of biomarker validation and translation from bench to bedside.

In recent years, significant advances in genomics increasingly impact disease detection, prognosis, prediction, and efficient patient stratification. Moreover, genomic biomarkers greatly influence the development of personalized medicine by providing treatments adapted to the genetic characteristics of the individual patient's disease.

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Endometriosis and Diagnostic Delay: The Patient's Perspective

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Abstract Early diagnosis is a top priority for women with endometriosis. However, diagnostic delays are still common. It is of utmost importance to prevent diagnostic delay as this can have an adverse impact on women's physical and psychosocial health, including undergoing unnecessary treatments and a worsening of symptoms over time. Furthermore, women with endometriosis experience a sense of relief at diagnosis. The factors contributing to diagnostic delay are well documented and are differentiated into patient and medical factors, such as normalization of symptoms form both sides. The next step is to conduct more research into preventing diagnostic delay and translating evidence into clinical practice by educating the general public on the topic of endometriosis, by monitoring healthcare quality and by conducting research into biomarkers.

Keywords Endometriosis • Patient centredness • Patient centricity • Adolescent endometriosis • Quality of life • Diagnosis • Diagnostic delay

Diagnostic delay in women with endometriosis refers to the time gap between the onset of symptoms and receiving a medically confirmed diagnosis. A myriad of studies reported that diagnostic delays are common in women with endometriosis. However, reported delays seem to be much longer in patients recruited from patient associations as compared to patients recruited from secondary and tertiary care clinics (i.e. a median delay of 7 years vs. a median delay of 1.5 and 2 years, respectively) [1].

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Early diagnosis is a top priority for women with endometriosis. A recent systematic review, including all studies on endometriosis patient's perspective on healthcare, concluded that there are three related primary targets for improving the patient centredness of care, namely: respecting patients, believing patients and timely diagnosis of endometriosis [2]. These three care aspects were unequivocally reported as important and problematic by at least five studies [2]. More specifically, regarding a timely diagnosis, five qualitative studies agreed on its importance to patients [3–7], and nine qualitative studies agreed that patients assessed the service quality problematic in this respect [3–11]. An example of a patient quote: *I think the biggest problem initially was getting a diagnosis which took years* [6]. A recent empirical study, questioning patients from a European clinic on the importance and service quality of 38 care aspects, confirmed the relevance of this target to improve the patient centredness of endometriosis care [12].

It is our job as healthcare professionals to diagnose patients with endometriosis in time as to preserve these women from many years of unnecessary suffering.

Why Is a Delay in Diagnosis Problematic?

Despite the reported differences in the length of diagnostic delay, the literature is unequivocal on the adverse consequences of a delayed diagnosis, both from a socio-economical perspective and a patient's perspective. From a socio-economical perspective, a timely diagnosis is important as it might reduce endometriosis-associated costs by decreasing productivity loss and healthcare consumption [13]. A multicentre study estimated the average annual total costs of endometriosis at €9579 per patient [14]. From a patient's perspective, the delay in diagnosis is detrimental to patients' physical and psychosocial health.

Regarding patients' physical health, the delay in diagnosis of endometriosis increases not only the length of suffering from symptoms but also its severity as symptoms worsen over time [15, 16]. For example, if endometriosis is already present but not diagnosed and treated during adolescence, it can progress and result in infertility during adulthood [17]. Moreover, a delayed diagnosis can also cause women to undergo many (unnecessary) medical tests and treatments [16]. Furthermore, receiving a timely diagnosis is important to start the correct treatment. Medical treatment is associated with alleviation of pain and improved pregnancy rates [18]. Surgical treatment can result in improvements of pain, quality of life and sexual functioning and is associated with good fertility rates [19, 20].

Regarding patient's psychosocial health, quantitative studies found an association between diagnostic delays and reduced health-related quality of life [21]. Furthermore, Staal et al. [22] found that the long time lag until recognition of endometriosis symptoms as part of a disease is a traumatizing experience for patients. Patients feel angry and frustrated for not being believed or understood by their healthcare professionals who normalized their symptoms as part of

menstrual pain or disregarded them as imaginary [3, 16, 23]. An example of a patient quote: *When they gave me my endo-diagnosis, I was fuming that I hadn't been believed and that I hadn't been taken seriously* [4]. Moreover, the fear of not knowing what is wrong (i.e. diagnostic uncertainty) [23] and the social stigma of not being able to perform as normal at work and at home add to the traumatizing experience [3].

Why Is Diagnosis a Relief?

When a diagnosis is reached, women will initially feel overwhelmed and worried as there is no cure and because of the risk for infertility [16]. Later on, receiving a diagnosis is experienced as a relief for the following reasons: (1) it provides a language to talk about symptoms (i.e. possibility to communicate about the disease to family or employer); (2) it provides a sense of legitimation and, thus, justified access to psychosocial support; (3) it makes feelings of fear and self-doubt disappear; (4) it gives a feeling of liberation and empowerment; (5) it offers hope for appropriate treatment and, hence, pain reduction; and (6) it means that the symptoms are not caused by a deadly disease, such as cancer [3, 16, 23]. An example of a patient quote: *I was in a way relieved because you have the answers like there was something wrong and that's why you are experiencing what you are, plus it wasn't sort of like an ectopic pregnancy and other things that I'd been really scared about up until being diagnosed. At the same time a bit overwhelming that it was something that was going to stay there, that it wasn't just something that could be treated with antibiotics. Plus the risk it has to your fertility and stuff like that* [16].

What Causes the Delay in Diagnosis?

According to a recent Belgian study, a significant delay between the onset of symptoms and the patient's initiative of seeking medical help exists (i.e. an average of 1 year for a total delay of 2 years) [13]. Indeed, Ballard et al. [3] studied the reasons for delay in diagnosis and differentiated patient factors (e.g. normalization of symptoms or feelings of embarrassment) from medical factors (e.g. normalization or hormonal suppression of symptoms or using non-discriminatory investigations). The following patient quote is exemplary for the medical factors: *From the time I was 13, I went through a number of different doctors to try and find the problem, most just told me that some people have heavier periods than others and more pain and don't cope well with the pain, and that it was normal* [16]. A recent study by Moradi et al. [16] pointed out that patient factors are also related to the patient's family, friends and colleagues. More specifically, women with endometriosis described that they normalized their symptoms amongst others because their family and friends told them that pain and bleeding were normal.

What Do We Still Need to Know?

Although the factors contributing to the diagnostic delay are well documented, many women still experience significant delays [23]. This means that the implications of previous research should be taken into account by clinical practice and that more research on preventing diagnostic delays is required. Patients will appreciate research into diagnostic delays. The participants from the study of Staal et al. [22] on diagnostic delay reported that this study helped them to emotionally deal with the disease as it made them feel understood and taken seriously.

First of all, in order to limit the medical factors causing the delay, the flaws in the current diagnostic process in case of pelvic pain should be identified. Currently, the knowledge on endometriosis and diagnostic strategies in women with endometriosis of Dutch general practitioners (GPs) are, for example, being investigated [22]. It would also be interesting to find out if diagnostic delays differ between countries with different healthcare systems.

Second, research efforts should be devoted to developing tools that aid diagnosis based on symptoms. Currently, a definitive diagnosis of endometriosis requires laparoscopy, but this invasive procedure cannot be performed on the entire population. A Scandinavian group of researchers has developed an anamnestic tool for physicians [24]. Our own group has developed a diagnostic self-screening tool [25]. Measuring menstrual pain and defining what is considered normal and what is not are still challenging. When pain begins soon after menarche, neither the patient nor the physician would know if the experienced pain is of a normal intensity or whether it was abnormal and was caused by a disease such as endometriosis [26].

Third, existing endometriosis-associated biomarkers should be validated, and new biomarkers should be identified to develop an accurate, noninvasive method to diagnose endometriosis [27]. An Iranian qualitative study found that both patients and physicians agree on the importance of reliable diagnostic indicators [28].

In order to limit the patient factors causing the delay, women with endometriosis have stressed the importance of increasing awareness and understanding in society about endometriosis, for example, at schools [16]. This is especially important since many women reported that they had not heard about endometriosis prior to their diagnosis [16].

Conclusion

Endometriosis still remains undiagnosed for years in many women, placing a significant physical and psychosocial burden on these women and an important socioeconomical burden on society. To prevent diagnostic delays, both patient and medical factors related to delay need to be addressed by educating the general public, monitoring healthcare quality and conducting research into biomarkers.

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Genetic Biomarkers for Endometriosis

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Abstract GWAS studies identified seven genomic regions with robust evidence for genome-wide significant association with endometriosis risk. One important question that arises is whether these genetic markers can be used to predict risk of developing endometriosis for individual women. As with most complex diseases, the effect sizes for genetic markers linked to endometriosis risk are small with odds ratios less than 1.3. If we combine information from all seven markers, we explain only 1.85% of the total phenotypic variance on the liability scale (assuming a population prevalence of endometriosis of 8%) with no predictive power for individual risk.

To explore the ability of all common genetic markers to predict endometriosis risk in individuals, we conducted simulations to quantify how useful endometriosis risk prediction is given current parameters. Applying our estimate of heritability ($h^2 = 0.26$) from all common SNPs and assuming data were available from ~30,000 endometriosis cases, the proportion of variance explained by the risk predictor is still only ~0.08. To improve this prediction would require a far greater sample size. Current data may be useful for population-based stratification into risk categories. This can have applications in some cases such as improved efficiency of screening in breast cancer. In the future, risk prediction for endometriosis might be improved through combining genetic risk scores with clinical data, estimates of environmental effects such as DNA methylation signals, and/or better understanding of disease subtypes.

Keywords Endometriosis • Genetic biomarkers • Risk • Prediction • Simulation

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Introduction

Endometriosis is a complex disorder influenced by multiple genetic and environmental factors. The genetic contributions to endometriosis risk are well documented, and several studies show that disease risk is higher among the relatives of endometriosis cases compared to controls in both hospital- [1, 2] and population-based [3] samples. This is further supported by twin studies showing an increased concordance in monozygotic when compared to dizygotic twins [4, 5] with the strongest evidence for genes influencing endometriosis from large-scale studies in twins [6] and in the Icelandic population [3].

Once the role for genetic variation was established, research efforts were directed toward identifying the genetic factors responsible. A large number of "candidate gene" studies have been published looking for association between endometriosis risk and genetic markers within biologically plausible candidate genes. In general, these studies were not successful with few results replicated or supported by later genome-wide association studies (GWAS) [7, 8]. Possible reasons for this have been reviewed elsewhere [7, 9]. Genetic linkage studies have also reported genomic regions that might harbor genetic variants increasing risk for endometriosis [10, 11], but no genes within these regions show significant association with disease risk.

In the last 5 years, the focus has shifted to large GWAS projects employing high throughput methods to genotype many thousand representative common genetic variants. This approach has revolutionized gene discovery for a wide spectrum of complex traits. Several GWAS studies for endometriosis have been published and results reviewed recently [8]. Seven genomic regions show genome-wide association with endometriosis with robust evidence across different populations and ethnic groups.

Given robust association between genetic markers and endometriosis risk, can these genetic markers be used to predict risk of developing endometriosis for individual women? Unfortunately, as with most complex diseases, the effect sizes for these genetic markers linked to increased endometriosis risk are small with odds ratios less than 1.3. Consequently, markers with robust evidence for association provide little power to predict a woman's risk of disease. More recently, methods have been developed to use genome-wide SNP genotype data for prediction. In this chapter we discuss genomic regions associated with endometriosis identified from GWAS studies and discuss prediction of individual risk from the associated markers and from genome-wide SNP prediction.

Genomic Regions Associated with Endometriosis Risk

Genome-wide association results have been published from four studies [12–15] and a meta-analysis [16] of summary data from the International Endogene Consortium study and the larger Japanese study. In addition, replication studies for

some of the key SNPs identified in the GWAS studies have been published [17–19]. There is excellent agreement across all studies for the major regions implicated in endometriosis risk [8]. Six regions showed evidence for genome-wide significant association in all cases, severe cases or both groups, and results for the region around fibronectin 1 (*FN1*) are close to genome-wide significance with strongest evidence in severe cases [8]. Recently, a meta-analysis of imputed data from Nyholt et al. [16] and the published results from Adachi et al. [12] confirmed association between endometriosis risk and SNPs in the region of interleukin 1A (*IL1A*) on chromosome 2 [20] adding a further important region for follow-up.

Heritability

Heritability (h^2) is an estimate of the proportion of variation in disease risk due to genetic factors. Traditionally this was estimated from similarities and differences in risk for relatives. One widely used design is to consider disease risks between pairs of identical and nonidentical twins. If the risk is higher for identical twins, this is evidence for a genetic contribution to disease risk since identical twins share 100% of their genome while nonidentical twins share only 50%. Using the classical twin design in a large sample of Australian twins, heritability for endometriosis risk was estimated at ~50% [6]. The remaining 50% of risk is due to other factors including environmental influences.

Studies trying to dissect the genetic and nongenetic causes using familial aggregation studies based on phenotypic observations alone must make explicit assumptions about shared environmental influences that are difficult to exclude entirely. More recently, whole-genome genotyping through GWAS provides an alternative method to estimate genetic contributions to disease risk independent of assumptions about shared environment necessary in family-based designs. We have used this method to estimate the genetic contribution to endometriosis from common genetic markers, sometimes called the SNP-heritability. After standard QC, the number of samples and SNPs used for estimating the genetic variance was 10,135 individuals (3154 cases and 6981 controls) with ~500,000 common SNPs [21]. We estimated that SNP-heritability on the liability sale was 0.26 (SE 0.04) assuming the population prevalence is 0.08 [21].

The difference between the SNP-heritability and heritability estimated from twin studies is likely due to several factors including uncertainty in the heritability from twin studies, the 500,000 common variants do not capture all the contributions from many rare variants, gene x environmental effects were not properly modelled, and possible heterogeneity from combining all endometriosis cases with different levels of severity and presentation. This difference between twin estimates of heritability and SNP-heritability is a general phenomenon for complex diseases. Many studies are now investigating those factors that can explain the discrepancy between the SNP-heritability and heritability. For the present study, the estimate of SNP-heritability

sets the practical upper limit for the ability of genetic markers to predict risk of disease. For endometriosis, this is ~25% of the variation and requires using data from genome-wide genotyping.

Genetic Architecture of Endometriosis

The genetic architecture for a disease or trait is defined as the number of loci affecting the trait, the distribution of effect sizes, interactions between the genes or loci, and interactions with the environment [22]. GWAS results provide strong evidence for genomic regions associated with endometriosis risk. However, association results must pass stringent thresholds for significance and be replicated in independent studies before risk variants are accepted as contributing to disease risk. Only a few of the "top hits" meet these criteria in most genome-wide studies. Many other variants lie just below the threshold. A proportion of these markers will be "truly" associated with disease, but cannot be distinguished from the other false positive signals.

Larger studies help to discover more of the risk variants, but the application of multivariate statistical approaches to the entire marker data set can also be used in other important ways to understand the nature of genetic contributions to disease risk. Genetic risk prediction (GRP) methods make use of the aggregate effects of many genetic variants where one data set serves as discovery sample, with association tested in a target set [23]. Variants of small effect (e.g., with genotype relative risk of 1.05) are unlikely to achieve even nominal significance in a GWAS analysis; however, increasing proportions of true effects will be detected at increasingly liberal *p*-value thresholds. In the discovery sample, sets of allele-specific scores are selected for SNPs with the different levels of significance (e.g., $P < 0.01$, 0.05, 0.1, 0.2, etc.). Genetic risk scores for individuals in the target set are then calculated as the sum of the copies of risk alleles for that individual in the target set weighted by the allelic effects (log odds ratio) estimated from the discovery set. The term risk score is used instead of risk, as it is impossible to differentiate the minority of true risk alleles from the nonassociated markers.

Applications of GWAS Data Beyond the Top Hits

Genetic profiles can be used in important ways to investigate the genetic architecture of endometriosis. Our results show that analyses of all SNPs in the endometriosis GWAS data sets provide powerful approaches to investigate subgroups of endometriosis and understand shared genetic contributions across studies [14, 16, 24].

It is often difficult to determine the relationship between disease classes with strongly overlapping symptoms. In genetic studies of endometriosis, the Revised American Fertility Society (rAFS) classification system is commonly used to stage disease severity and assigns patients to one of four stages (I–IV) on the basis of the

extent of the disease and the associated adhesions present [7, 8]. Other classification systems have been proposed including ovarian vs. peritoneal disease and deep infiltrating vs. superficial disease. Whether these subclasses represent the natural history of one disorder, or are in fact different disease subtypes, is an important consideration in endometriosis research. Analysis of genome-wide marker data can assess the genetic contribution to individual disease subclasses and also the shared genetic contribution to each subclass providing new insights into the different disease presentations [24].

We have applied genetic risk prediction methods to show a stronger genetic contribution to severe disease compared with minimal/mild cases of endometriosis [14]. Further analysis of different disease classes [24] confirms the stronger genetic association with severe disease. In addition, mild forms of the disease in the discovery sample predict milder forms of disease in the target sample, but not more severe forms. Larger samples will be needed to confirm this result, but the data suggest distinct genetic contributions to mild forms of the disease. Similar methods also show strong genetic overlap for endometriosis cases in both European and Japanese populations [16].

Taken together, results from the GWAS, estimates of SNP-heritability, and polygenic prediction methods demonstrate that genetic contributions to endometriosis risk are due to a large number of common variants each with small effects. No common variants with large effects have been detected. Genome-wide significant "hits" all have small effects (odds ratios <1.3), and many more genetic variants affecting disease risk remain to be discovered.

Risk Prediction for Endometriosis

As noted above, the individual risks conferred by markers showing genome-wide significant association with endometriosis are low and do not help with prediction of individual risk. Even if we combine information from loci discovered from GWAS, they still have poor predictive power to discriminate individual risk. We combined results for the seven genome-wide significant loci from data on the reference allele frequencies and odds ratio from meta-analysis of the combined Australian and UK samples including 3181 case and 8075 controls [16]. Using a liability threshold model [25], the variance explained by the seven SNPs was 1.85% of the total phenotypic variance estimated on the liability scale assuming a population prevalence of 8%.

To explore the ability of all common genetic markers to predict endometriosis risk in individuals, we conducted simulations to quantify how useful endometriosis risk prediction is given current parameters. In this case, data from a large discovery sample are used to rank markers positively associated with endometriosis risk and develop a marker set which, when the markers are genotyped in an individual, would provide some prediction of disease risk. The accuracy of the prediction depends on a number of parameters and is strongly influenced by the size of the discovery sample.

Prediction Accuracy and Sample Size

Using recent results on prediction accuracy of polygenic scores derived from quantitative genetic theory [26, 27], we quantified the relationship between sample size of the discovery sample and prediction accuracy. We assumed that endometriosis was polygenic [14, 16], the population prevalence was 0.08, and heritability on the liability scale was either 0.26 [21] based on SNP-heritability or 0.5 [6] from twin studies. We further assumed that the proportion of cases in the discovery sample was ~30% (~twice the number of controls compared with the number of cases) and 8% for validation set (i.e., population sample). The effective number of SNPs was assumed to be 50,000 [28].

Results show that when the heritability is $h^2 = 0.26$ (Fig. 1), the proportion of variance explained by the risk predictor is ~ 0.08 even with 101,350 individuals (31,540 cases and 69,810 controls). However, the same proportion of variance can be achieved with ~20,270 individuals when heritability is $h^2 = 0.5$. A similar pattern is observed for the area under the curve (AUC; Fig. 2). An AUC of 0.65 requires 101,350 individuals with $h^2 = 0.26$, but requires only 20,270 individuals with $h^2 = 0.5$.

Following a common epidemiological approach to assess a continuous risk factor [23, 29], individuals were stratified into deciles according to the ranked values of the genetic risk predictors. We quantify the odds ratio of case-control status by contrasting the top decile to the lowest decile (Fig. 3). This approach is powerful even with a relatively small discovery sample, indicating this may be a valuable tool to stratify a heterogeneous population into groups.

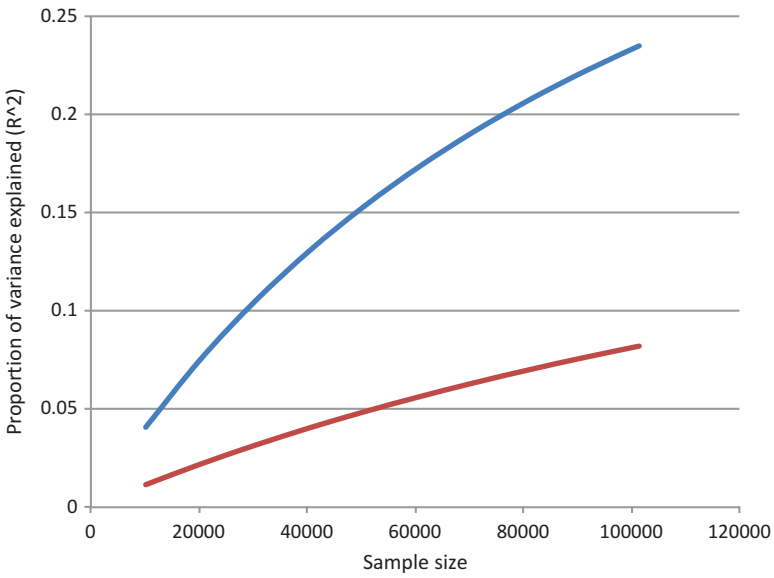

Fig. 1 The proportion of variance in endometriosis risk explained (R^2) is plotted against sample size for the discovery sample. The *red line* assumes $h^2 = 0.26$, and the *blue line* assumes $h^2 = 0.5$

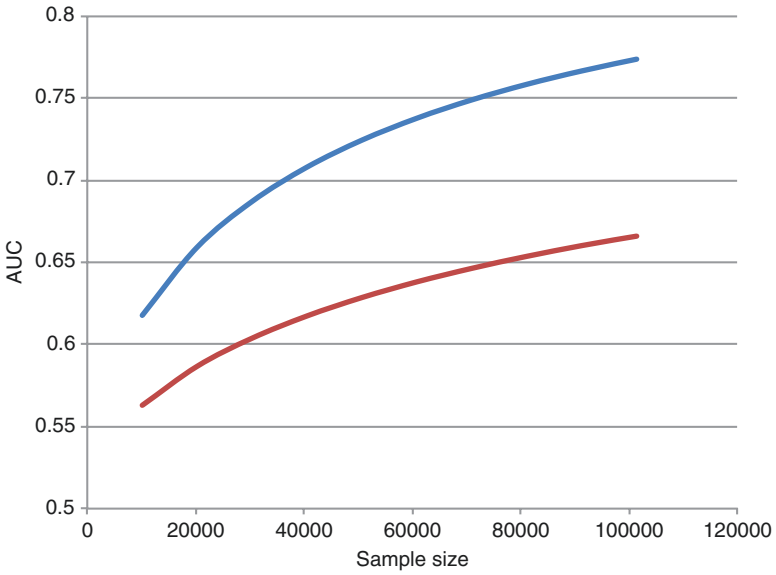

Fig. 2 Area under ROC (receiver operating characteristic) curve (AUC) plotted against sample size. The *red line* assumes $h^2 = 0.26$, and the *blue line* assumes $h^2 = 0.5$

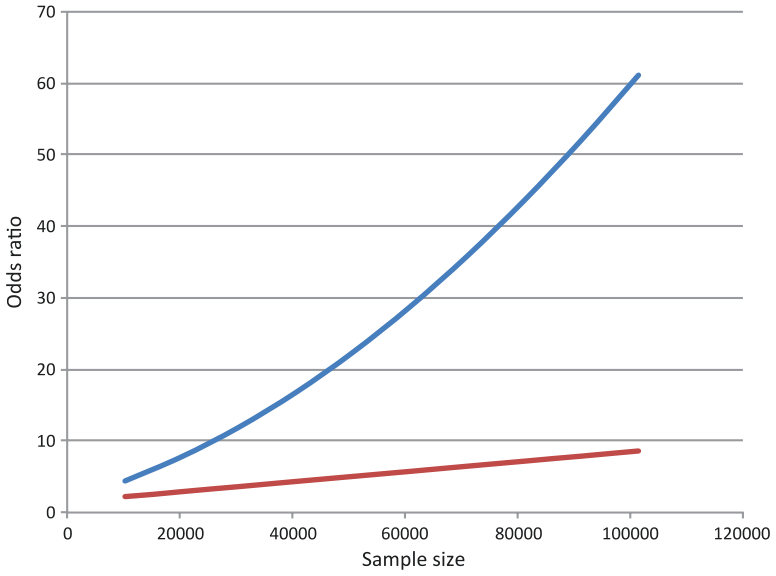

Fig. 3 Odds ratios of individuals stratified into deciles based on genetic risk predictors in validation data set, using the decile with the lowest risk as the baseline, plotted against sample size. *Red line* with $h^2 = 0.26$ and *blue line* with $h^2 = 0.5$

Summary and Future Directions

Current genome-wide significant "hits" provide no power for prediction of endometriosis risk for individual women. Studies of the genetic architecture of endometriosis and comparison with other complex diseases show that the genetic contribution to endometriosis is due to a large number of genetic variants each with small effects. The genome-wide significant "hits" represent only those markers that pass the stringent threshold required to account for the multiple testing required in GWAS analyses. Many other variants will be associated with endometriosis risk among the top SNPs that do not exceed the threshold and can provide useful information for prediction.

As we show in our simulations, the precision of genetic risk predictors constructed from a discovery sample depends on the size of the discovery sample and heritability of the disease. A very large discovery sample will be necessary to develop genetic risk scores with any accuracy for prediction. The meta-analysis of the International Endogene Consortium and Japanese GWAS studies analysed 4604 endometriosis cases. A new consortium of international groups is assembling data for ~17,000 cases and a large number of controls. Even with a discovery sample of this size, genetic risk predictors will still only explain a small proportion of variance in disease risk at SNP-heritability of 0.26.

Future developments may improve the prospects for including genetic markers in predictive tests for endometriosis risk. Risk prediction is an active area of research and a number of groups are working on ways to improve prediction estimates [30]. Although genetic markers do not provide accurate estimates for individual risk prediction, we show that current estimates may still be useful for population-based stratification into risk categories. This approach is being considered in breast cancer screening where including risk scores could change the current recommendations based on age [31]. Inclusion of risk scores could allow younger women with equivalent absolute risk to benefit from screening while decreasing by ~25% the proportion of women in current age groups where screening is considered useful [31].

Risk estimates from genetic marker data could be combined with clinical information to improve prediction. In breast cancer, addition of risk estimates from marker data for seven loci gave a small improvement in risk prediction based on family history, reproductive information, environment, and lifestyle factors [30]. Another consideration is that the current GWAS "hits" are unlikely to be the functional variants [32]. Identifying the true functional variants at each locus may improve the accuracy of risk estimates.

Risk prediction may vary across different disease subtypes. Current GWAS studies in endometriosis include cases of clinically diagnosed and self-reported disease and are combined across all disease stages. We have shown that the genetic architecture may differ between mild and severe forms of the disease [24]. If this is the case, separation of cases into meaningful subtypes may improve the precision of risk predictors within subtypes. However, very large studies will be necessary to achieve appropriate power for the different subtypes.

Endometriosis is influenced by genetic variation and also by environmental factors. One promising approach being used in other complex traits is the study of genome-wide methylation signals [33, 34]. Methylation signals are themselves influenced by genetic variation [32], but they also capture past and present environmental effects [34]. As we have seen, the accuracy of risk prediction depends on the disease heritability. Risk prediction in endometriosis would be improved if the heritability explained by genetic markers was nearer to the estimate ($h^2 = 0.5$) from twin studies [6]. Even if we can account for all of the genetic variation, this still leaves half of the variance in endometriosis risk unexplained.

One approach we are following up is whether genome-wide methylation can capture some of the environmental influence and be used to improve disease prediction. Using similar approaches we have evaluated combining genetic risk scores and methylation risk scores for prediction in studies on body mass index (BMI) and height [35]. BMI has modest heritability and is influenced by environment, while height has very high heritability. Combining risk scores from GWAS and methylation substantially increases prediction for BMI but does not improve prediction for height.

In conclusion, genetic variants associated with endometriosis risk do not provide useful markers to predict individual risk for endometriosis, whether restricting the markers to genome-wide significant results or combining data into polygenic risk scores. Much larger genetic studies will be required to approach useful prediction. There are promising developments to improve prediction through combining genetic data with other data. This includes clinical data and predictors from genome-wide methylation signals. Further studies will be required to determine if these approaches are useful for endometriosis risk prediction.

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Epidemiological and Clinical Risk Factors for Endometriosis

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Abstract The etiology and risk factors associated with endometriosis are unclear. Over the recent decades substantial work has investigated epidemiological risk factors for the disease. While some clear patterns emerge with regard to risk, many studies are plagued with complex methodological issues and bias. This chapter will present the current state of the epidemiologic knowledge on risk factors for endometriosis including in utero factors, menstrual and reproductive factors, lifestyle factors, physical characteristics, anthropometric factors, dietary factors, and environmental factors. It will further explore the relationships between endometriosis and other chronic diseases. Future work should aim to fill gaps in knowledge while taking the complex methodological issues into account.

Keywords Endometriosis • In utero • Menstruation • BMI • Physical activity • Diet • Study design • Methods • Cancer • Environmental toxins

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Prevalence and Incidence

The prevalence and incidence of endometriosis in the general population are difficult to quantify because of the need for surgery to establish a diagnosis. Estimates vary significantly between different population samples and modes of diagnosis. For example, among asymptomatic women seeking tubal ligation, the prevalence of incident endometriosis was found in two studies to be between 2 and 18% [1, 2] with an average prevalence of 4% [3]. Conversely, among women presenting with pain or infertility, endometriosis prevalence has been reported to range from 5 to 50% within infertile populations [4–10] and between 5 and 21% in women hospitalized for pelvic pain [5–8]. A recent investigation, the ENDO study, aimed at better understanding the incidence of endometriosis. They enrolled 495 women into an operative cohort of women undergoing laparoscopy/laparotomy between 2007 and 2009 and 131 women into a cohort from the general population near the surgical centers. They found that among women scheduled to undergo laparoscopy, approximately 41% had endometriosis compared to approximately 11% among an unscreened population sample of women [11].

Among the few studies that investigated adolescents with severe dysmenorrhea, approximately 50–70% are diagnosed with endometriosis [12–14], and this number has been shown to be as high as 70% in those who did not respond to conventional medical therapy [15]. However, adolescents that undergo laparoscopic surgery may have more severe pain symptoms than adults undergoing the same procedure. Thus the prevalence estimated among symptomatic women undergoing surgical procedures will be higher than in the general population, while the prevalence derived from asymptomatic populations undergoing elective tubal ligation is likely to underestimate the public health burden of disease. Extrapolating prevalence rates for pelvic pain and subfertility in the general population, and observed average diagnostic rates of endometriosis in these groups in clinical settings, the estimated prevalence of all-stage endometriosis is 5–10% and ~ 2% for moderate/severe disease (rAFS III/IV) [16]. No autopsy data for any age group has been published to date.

Incidence data in the general population are less readily available. Incidence rates were first reported from a hospital-based cohort study by Houston et al. [17]. The incidence of histologically confirmed endometriosis among white 15–49-year-old women in Rochester, Minnesota, between 1970 and 1979 was 160/100,000 person-years (PY). Incidence increased with age, from 17/100,000 PY among women aged 15–19 to 285/100,000 PY among those aged 40–44. The incidence rate fell to 184/100,000 PY among women aged 45–49. In an updated study from 2004 within the same geographic region, Leibson et al. reported an overall incidence of clinically diagnosed endometriosis of 187/100,000 between 1987 and 1999. The recent rate estimate was the highest in a younger population among women aged 25–34 (380/100,000 PY) and lowest for those over 54 years (16/100,000 PY) [18]. These differences may be indicative of changes in diagnosis and health-seeking behavior between generations.

Similar patterns to Leibson were found using data from the Nurses' Health Study II (NHSII), a prospective nationally representative cohort of 116,430 US female

nurses aged 25–42 at time of enrollment in 1989. The 10-year incidence rate of laparoscopically confirmed endometriosis was found to be 298/100,000 PY [19]. Incidence varied between infertile and non-infertile women. The incidence rate among women with no prior infertility was 237/100,000 PY and was noted to decrease significantly as women reached their late thirties to early forties. Among women with a history of infertility, the age-adjusted incidence rate of laparoscopically confirmed endometriosis was 1,380/100,000 PY. While the overall incidence of endometriosis decreased with increasing age regardless of infertility status, the decrease in risk was more modest among women who never reported infertility, declining only after age 44 (trend p-value <0.0001) [19]. Both of these studies suggest that endometriosis diagnosis occurs primarily during the childbearing years. The complexities in the endometriosis case definition, delay between symptom onset and disease diagnosis, and appropriate study population will be discussed in more detail later.

Hypotheses Regarding the Etiology of Endometriosis

The pathogenesis of endometriosis is complex and multifactorial. The earliest theory of the etiology of endometriosis was established in 1927. Dubbed "Sampson's theory," it posits that retrograde menstruation and subsequent implantation and growth of endometriotic tissue on extrauterine structures are the primary causes of endometriosis [20]. This theory is supported by studies demonstrating clustering of endometriotic lesions around the distal ends of the fallopian tubes and the presence of viable endometrial cells in peritoneal fluid [21] and factors implying increased exposure to menstruation (earlier age at menarche, shorter cycle length, heavy flow) increases endometriosis risk. However, a majority of women experience retrograde menstruation in some capacity. It was estimated that 90% of women experience retrograde menstruation [22], suggesting that the true differences between women with and without the disease may be due to varying rates of implantation of endometrial cells and not the occurrence of retrograde menstruation itself. Factors that influence adherence, proliferation, and maintenance of the cells and lesions (such as hormonal milieu, immunological factors, and angiogenic processes) have been implicated.

Certain hormonal environments, in particular exposure to estrogen, may facilitate the proliferation and survival of endometriotic tissue. There is a wide range of circumstantial evidence that shows that endometriosis risk factors are also associated with hormone levels. For example, the association between endometriosis and age at menarche, body mass index (BMI), and oral contraceptive use, as well as the prevalence of the disease among reproductive aged women, all suggest a hormonal association. Additionally, early work has shown that endometriosis plaques have estrogen, progesterone, and androgen receptors and grow in the presence of estrogen but atrophy when exposed to androgens [23–26]. This theory is not inherently independent of the retrograde menstruation theory, since hormone levels may influence the volume of retrograde menstruation or the promotion and survival of endometrial implants outside of the uterus.

In addition, there is evidence to suggest that endometriosis is associated with immunologic and inflammatory responses [27]. Women with compromised immune systems may have more endometrial plaques outside of the uterus than women with normal immune function. Case-control studies have observed abnormal levels and function of growth factors, macrophages, and pro-inflammatory cytokines in the peritoneal fluid and serum of women with endometriosis [9, 23, 25, 28, 29]. Several case reports and small studies suggest an increased risk of autoimmune diseases among women with endometriosis [30–36]. These observations prompted a cross-sectional study of a potential association between endometriosis with periodontal disease—another chronic inflammatory disease more common in women with systemic autoimmune disorders [37]. Women with self-reported endometriosis were observed to have significantly higher odds of having both gingivitis and periodontitis (odds ratio [OR], 1.6; 95% confidence interval [95% CI], 1.1–2.3), suggesting a possible association between the two inflammatory conditions. The insulin-like growth factor (IGF) system has also been suggested in the pathogenesis of endometriosis. However the literature in humans has been mixed, with early case-control studies suggesting a positive association between IGF-1 levels and endometriosis [38, 39], but more recent studies finding no association [40–42].

Methodological Issues

Several methodological issues complicate the study of endometriosis including defining the disease, choosing an appropriate comparison group, and adequately capturing the appropriate etiologic window for disease onset [16, 43–46]. While these issues can plague all study designs, case-control studies may be more vulnerable to bias due to issues with control selection and recall.

Endometriosis case definition: The selection of an appropriate case definition can be challenging because the current clinical definition of endometriosis includes a wide spectrum of symptoms and pathologic findings. The few epidemiologic studies that were able to evaluate the severity of endometriosis based on the American Society for Reproductive Medicine (ASRM) disease staging among cases do not demonstrate more extensive disease among women with laparoscopic confirmation [47]. However, genetic studies have shown that stage III/IV disease is etiologically different from stage I/II disease [48].

Laparoscopy has long been considered the gold standard for endometriosis diagnosis [49, 50] as accuracy of self-reported endometriosis in the absence of laparoscopic confirmation has been poor [47, 51]. Limiting the definition of endometriosis to those with surgical confirmation, however, may introduce selection bias. It is possible that patients with more frequent utilization of the medical system or those with the most symptomatic disease may be more likely to undergo laparoscopy and that women whose symptoms improve with less invasive treatments such as anti-inflammatory medications or oral contraceptives may not seek an invasive, albeit confirmatory, diagnosis. Defining endometriosis as the presence of functional endo-

metrial glands and stroma outside of the uterus allows women who have asymptomatic disease noted at the time of unrelated surgery, such as tubal ligation, to be included as cases. Public health investigations are primarily interested in symptomatic disease resulting in morbidity for the patient; thus it has been suggested that endometriosis be defined not only by the presence of ectopic endometrium but also by evidence that the lesions are active and have affected normal physiology.

Selection of cases that receive an endometriosis diagnosis from an evaluation for infertility may similarly under-sample those with pelvic pain. Had such women not attempted to become pregnant or had not had access to medical help with infertility, most would never have come to a laparoscopic diagnosis of endometriosis. Endometriosis discovered as a cause for infertility may differ from endometriosis diagnosed from pelvic pain. Additionally, there is concern that endometriosis cases sampled are more likely to be prevalent, and thus an individual may have already changed many lifestyle factors in response to undiagnosed disease creating the potential for reverse causation in the study of many risk factors.

Appropriate comparison group: An important challenge in studying endometriosis is determining the appropriate comparison group. In case-control studies, no strategy for control selection appears entirely satisfactory because factors that might influence which women receive a diagnosis of endometriosis may also be related to exposures of interest. Controls must represent the exposure distribution of the population which gave rise to the cases, and sampling must be independent of that exposure. Any restriction or exclusion applied to cases must also be applied to controls. To date, control selection has largely focused on preventing the inclusion of undiagnosed cases in the control group in an effort to reduce misclassification. As a result of the invasive nature of diagnosis, studies have often chosen controls from groups of women who have had pelvic surgery for other reasons (e.g., tubal ligation, hysterectomy, or laparoscopy for reasons other than endometriosis). It is likely that these highly selected women represent a biased sample of those from the underlying population, e.g., by definition, tubal ligation controls are highly likely to be fertile and have had children [16, 43].

When the cohort or case and control groups are comprised of both infertile and non-infertile women, analyses should be conducted first with all women and then stratified by fertility status. When this stratification is not possible, researchers must remember that comparing cases to infertile controls may yield results that differ in interpretation from those that would be observed when comparisons are made to a control group of fertile women without endometriosis. Women who seek a medical evaluation or treatment for infertility differ on important demographic, lifestyle, and access factors from infertile women who do not utilize these services; thus failure to take these differences into account may lead to selection bias or generalizability issues [52]. This is particularly important when the exposures of interest, such as menstrual cycle characteristics or reproductive history, are correlated with both endometriosis and infertility.

Another concern in choosing a comparison group is detection bias. The precision of evaluation may differ between cases identified during a workup for infertility or pelvic pain and controls who were declared to be free of endometriosis during a

surgical procedure not initiated by symptoms of the disease [53]. A purely population-based selection of controls has also been argued against, because women with undiagnosed disease may be selected, thus attenuating the association between exposure and disease. The impact may be minimal, however, as the community prevalence of asymptomatic endometriosis is low [16].

Onset of endometriosis symptoms: Epidemiologic studies should ideally focus on incident rather than prevalent cases of disease. However, chronic conditions like endometriosis are typically diagnosed only after a symptom threshold is reached, making identification of the exact time of onset of disease impossible. This matter is additionally complicated by significant delay from endometriosis symptom onset to diagnoses. According to the Endometriosis Association's 1998 North American Member Survey, the average time to diagnosis from symptom onset is 9.3 years, with women waiting an average of 4.7 years to seek clinical help and another 4.6 hears from first clinical visit to formal diagnosis [34]. Recent estimates have reported similar delay ranges [54–56]. Consequently, most analyses are in reality estimating the incidence of endometriosis *diagnosis* rather than the true incidence of disease onset, and the temporal relation between exposure and disease must be interpreted critically. There is great concern when studying modifiable risk factors that women may change their behavior after onset of endometriosis symptoms but before an endometriosis diagnosis.

It is through this lens of understanding the methodological issues that we may appreciate the complex nature of endometriosis research and more accurately interpret studies and risk factor burden.

Risk Factors

In Utero Environment

With the advent of the Barker hypothesis of the fetal origins of chronic disease [57], there has been increasing interest in the impact of the uterine environment on disease risk [57, 58]. The in utero environment may reflect maternal exposures and hormonal and inflammatory states that may have important implications for disease risk later in life.

Birth weight may reflect the maternal hormonal milieu or adequacy of uteroplacental blood flow during the pregnancy. Within the NHSII cohort, women born at lower birth weight (defined as 5.5 pounds) were at a significantly greater risk of endometriosis compared to normal or high birth weight women (7.0–8.4 pounds) (p-value, test for linear trend = 0.01) [59]. In addition, women who were born as one of a multiple gestation were at greater risk of endometriosis, even after controlling for birth weight (risk ratio [RR], 1.7; 95% CI, 1.2–1.5).

More recently, a smaller case-control study, the ENDO study, found that women with low birth weight (<5.5 pounds) compared to 7.0–8.4 pounds had increased, but

non-statistically significant, odds of endometriosis diagnosis (OR, 1.10; 95% CI, 0.92–1.32) [60].

Maternal exposure to exogenous environmental exposures has also been explored in relation to endometriosis diagnosis. In NHSII, women exposed to diethylstilbestrol in utero had a higher risk of endometriosis (RR, 1.8; 95% CI, 1.2–2.8) [59]. A prospective hospital-based study of 84 women of reproductive age noted a significant reduction in the odds of endometriosis for self-reported intrauterine cigarette exposure both in the absence (OR, 0.2; 95% CI, 0.1–0.8) and presence (OR, 0.1; 95% CI, 0.01–0.4) of current smoking [61]. A significant attenuation of risk of endometriosis was also observed among women who reported parental smoking behavior during gestation in the ENDO study [60]. Since epidemiologic results indicate that women who smoke cigarettes have lower levels of circulating estrogen [62], these findings support the idea that circulating maternal hormone levels are of importance in endometriosis etiology. No consistent statistically significant associations have been reported with intrauterine exposure to alcohol or caffeine and a diagnosis of endometriosis.

Menstrual and Reproductive Factors

Earlier age at menarche [47, 53, 63–65] and shorter menstrual cycles [1, 47, 65–71] have both been reported to influence the risk of endometriosis. A recent meta-analysis of 13 case-control studies looking at the association between early age at menarche (<12 years) and risk of endometriosis has been conducted to pool endometriosis data from multiple sources. When restricting the analysis to studies less prone to bias, they found that earlier menarche was associated with a statistically significant increase in a woman's risk of endometriosis [72]. Early age at menarche may increase frequency and duration of exposure to retrograde menstruation and may also reflect an altered hormonal environment. There is less consistent evidence related to monthly duration and heaviness of menstrual flow and tampon use. Various studies exploring these factors in relation to risk of endometriosis have reported a direct association [63, 65], no association [66], and an inverse association [73, 74].

While pregnancy may serve as an important etiologic and detection window for endometriosis, methodologically it is difficult to study. Hormonal changes during pregnancy and lactation may prevent or diminish the growth of endometrial explants. Case-control studies [68, 70, 75] and one cohort study [47] have shown a decreased risk of endometriosis among women with greater parity. Additionally, a strong inverse association between duration of lactation and endometriosis risk was observed (RR, 0.2; 95% CI, 0.1–0.4 for women who breastfed >23 months compared to women who never breastfed) [47]. While the benefit from lactation was lost 5 years after delivery, the effect of pregnancy appeared to remain constant regardless of time since last birth. The protective association of pregnancy may be caused by endometrial decidualization and cervical dilation during childbirth, which may diminish the volume of retrograde menstruation once menses resume postpartum.

Methodologically, childbearing is of interest because endometriosis is a cause of infertility in some women. Additionally, medical evaluations for infertility may find "asymptomatic" endometriotic lesions. Thus there are important issues related to diagnosis of endometriosis during childbearing years. Data from the NHSII found that while women with endometriosis were at a twofold increased risk of incident infertility, among all women with endometriosis, 83% were parous by the age of 40 [76]. Similar findings have been reported in the ENDO study [77]. While women with endometriosis experienced a longer time to pregnancy than woman without endometriosis, irrespective of lesion staging, the vast majority of women with endometriosis who reported having planned a pregnancy also reported a successful live birth (87.5%).

Use of oral contraceptives (OCs) has been considered as both a risk modifier and a treatment for endometriosis pain. Oral contraceptives are usually prescribed as first-line therapy if endometriosis is suspected in adolescents [78]. Since oral contraceptive use may modify pain symptoms associated with endometriosis, it has been considered both an independent risk factor and a means to delay disease diagnosis. After pooling data from 18 studies (6 cross-sectional, 7 case-control, and 5 cohort) addressing oral contraceptive use and endometriosis, a uniform reduction in risk of endometriosis diagnosis for current oral contraceptive users compared to the comparison group was found (RR, 0.63; 95% CI, 0.47–0.85) [79] which was consistent with previous summaries [80]. They also found an increased risk of endometriosis for past users (RR, 1.21; 95% CI, 0.94–1.21). Similar patterns have been replicated in other cohort studies of oral contraceptives and with intrauterine devices [69, 81, 82]. Taken together, these findings suggest that current oral contraceptive use may improve pain among patients with prevalent endometriosis, therefore postponing diagnosis of the disease [83].

Physical Characteristics

Endometriosis has been found to be associated with specific physical phenotypes. Cross-sectional studies suggested the proportion of women with naturally red hair is greater among patients with endometriosis as compared to the general population [84–86]. While prospective data from the NHSII failed to demonstrate an overall statistically significant relationship, the authors observed an increased rate of endometriosis among women with naturally red hair who had never been infertile (incidence rate [IR],1.3; 95% CI, 1.0–1.7) and a decreased rate among those with concurrent infertility (IR, 0.4; 95% CI, 0.2–1.2) [87].

A large French nested case-control study of 4241 cases of surgically confirmed endometriosis (the E3N study) noted a positive dose-effect relation between risk of endometriosis and skin sensitivity to sun exposure (OR, 1.2; 95% CI, 1.1–1.4 for highest vs. lowest tertile), number of nevi (OR, 1.6; 95% CI, 1.4–1.8 for highest vs. lowest quartile), and degree of freckling (OR, 1.1; 95% CI, 1.0–1.2 for highest vs. lowest tertile), but, interestingly, no association with complexion or hair color [88].

The NHSII also found endometriosis risk was increased with presence of nevi on the lower legs (RR, 1.08; 95% CI, 1.02–1.14) higher level of skin's burning reaction to sun exposure in childhood/adolescence (RR, 1.20; 95% CI, 1.06–1.36) and family history of melanoma (RR, 1.13; 95% CI, 1.01–1.26) [89]. These findings are supported by an Italian case-control study related to skin's burning reaction tothe sun [90, 91]. Blue/green eyes have been shown to increase overall risk of endometriosis and also deep-infiltrating endometriosis [90, 92]. The relationship between endometriosis and phenotypic differences is posited to be through shared genetic factors, but the nature of this commonality remains to be explored.

Anthropometric Factors

Body size across the life course may influence endometriosis risk. The literature has consistently suggested an inverse association between endometriosis and adult body mass index (BMI) [19, 53, 63, 66, 91, 93–97]. However, the relationship with childhood and adolescent body size is not conclusive. In the NHSII, childhood body size estimated using Stunkard childhood body size images (averaged between ages 5 and 10) was inversely associated with risk of endometriosis diagnosis later in life (*P*-value for trend, 0.0002) [98]. Although there are known associations between childhood BMI and both age at menarche and adult BMI—both of which are known risk factors for endometriosis—the results of this study suggest an association between childhood BMI and endometriosis that was independent of these factors.

A case-control study using the same Stunkard childhood body size metric as the NHSII found a similar inverse association with early body size and endometriosis [96]. However, one recent case-control study using self-reported recalled childhood BMI noted a different result: women who reported being overweight at age 10 had an increased risk of endometriosis (OR, 2.8; 95% CI, 1.1–7.5) [99]. However, the body of evidence suggests an inverse relationship with adolescent body size and endometriosis [91]. Work using endometriosis cases from a Korean hospital found that body size was associated with the staging of endometriosis: more severe stages (III and IV) were associated with smaller childhood body size compared to less severe cases (*P*-values for trend, 0.002) [100].

It is interesting to note that some research has shown that genetic variants known to be associated with BMI were not found to be associated with endometriosis risk [101]. This may suggest that the association between BMI and endometriosis may be operating through common environmental factors or through a poorly understood selection bias mechanism.

Three case-control studies have reported an increased likelihood of endometriosis with taller height [53, 66, 96], although no association was found in the NHSII cohort study [19, 97]. The impact of body fat distribution on endometriosis has also been examined as women with a higher ratio of estrogens to androgens have been found to have increased peripheral fat accumulation [102]. A case-control study

found that the odds of surgically confirmed endometriosis was inversely related to waist-to-hip ratio (OR, 6.2; 95% CI, 1.4–26.7) for women <30 years old with a ratio of 0.6–0.7 compared to 0.8–1.0 [103]. A similar pattern of significant decreased risk was observed in the NHSII among women with the smallest waist to hip ratios of <0.6 compared to 0.7–0.79 [97]. In a meta-genome-wide association study of individuals of European ancestry, it was observed that an intergenic locus on 7p15.2 was significantly associated with both endometriosis and fat distribution (waist-to-hip ratio adjusted for BMI) [104].

These findings may support a prevailing etiologic hypothesis that endometriosis is influenced by circulating steroid hormones. It has been speculated that childhood body size and adult BMI may represent differences in insulin resistance and levels of sex hormone-binding globulin or insulin-like growth factors (IGF) during childhood and adulthood, which may influence endometriosis risk independently of age at menarche [98]. However, as summarized above, this relationship between endometriosis and IGF in adulthood has not been consistent across studies [41]. Childhood body size appears to be working independently of age at menarche although the mechanism is not firmly established.

Lifestyle Factors

While modifiable lifestyle risk factors are of great interest to the public health community, the study of these factors is complicated by potential changes in lifestyle following onset of endometriosis symptoms. This potential bias will be more pronounced in studies that depend on recall and studies that include both prevalent and incident cases.

The relationship between smoking and endometriosis is inconsistent. Some studies exploring the effect of smoking on endometriosis have found an inverse association [19, 63, 66], while others have found no association [19, 53, 67, 70, 81]. A study of adolescent exposure found indoor exposure to passive smoking during childhood increased risk of endometriosis diagnosis later in life (RR, 1.34; 95% CI, 1.09–1.64 with several hours exposure a day) [105]. While smokers have lower estrogen levels [62], they are also exposed to higher levels of exogenous estrogen in the form of dioxin, potentially complicating the association. Although data vary by tobacco source, it is estimated that a person who smokes a pack of cigarettes per day takes in about 4.3 pg of polychlorinated dibenzodioxins/kg body weight/day [106].

Studies have also examined whether caffeine and alcohol consumption increase the risk of endometriosis. Moderate intake of alcohol has been shown to increase total and bioavailable estrogen levels [107]. Studies of endometriosis within infertile populations have suggested a direct relation with caffeine and alcohol [108–111]; however, case-control studies including both infertile and fertile controls have observed no association [53, 65, 93, 94]. The NHSII observed no association with caffeine and an inverse association between alcohol and endometriosis (current alcohol intake >10 g/day vs. none; RR, 0.7; 95% CI, 0.6–0.8) [19]. A recent meta-analysis on caffeine consumption pooled data from 8 studies (6 case-control studies

and 2 cohort studies) and found no increased risk from caffeine or coffee intake (RR 1.26 and 95% CI 0.95–1.66 and RR 1.13 and 95% CI 0.46–2.76, respectively).

Physical activity may be associated with endometriosis; however, the data are conflicting [112]. Physical activity is thought to increase levels of sex hormone-binding globulin (SHBG) [113], lower luteal estrogens [114], and decrease insulin resistance and hyperinsulinemia [115, 116]. Two cohort studies of childhood/adolescent physical activity have shown increased endometriosis risk with high exercise levels [105, 117]. Conversely case-control studies of adult endometriosis have observed a 40–80% reduction in likelihood of endometriosis in regular as compared to non-regular exercisers [53, 66, 111, 118]. However, a recent analysis of the NHSII suggests a more modest, nonsignificant decrease in risk (RR, 0.9; 95% CI, 0.8–1.0) [119]. The slight inverse association between exercise and endometriosis was limited to women with no history of infertility. The prospective cohort design may more accurately estimate the temporal relation between physical activity and endometriosis risk; if women with endometriosis experience symptoms that restrict their ability to exercise, symptom-free women will predominate the physical activity group, resulting in an incorrect spurious protective effect.

Two case-control studies have explored the association between occupation and endometriosis [120, 121]. There is evidence that night shift work disrupts circadian estrogen secretion and increases risk of estrogen-dependent conditions such as breast cancer [122–124]. In a population-based case-control study of laparoscopically confirmed endometriosis, women who worked more than half of their job hours during night shifts demonstrated an increased odds of endometriosis (OR, 2.0; 95% CI, 1.0–3.9) [120]. A subsequent larger case-control study by the same group observed an increased odds of endometriosis in women who had ever worked as a flight attendant (OR, 9.8; 95% CI, 1.1–89.0), service station attendant (OR, 5.8; 95% CI, 1.0–32.4), or health worker (OR, 1.5; 95% CI, 1.0–2.2) [121]. In the NHSII, risk was elevated among women with concurrent infertility and ≥5 years of rotating night shift work (RR, 1.71; 95% CI, 1.18–2.49), compared with women with no rotating night shift work [125].

Dietary Factors

While there are a growing number of patient-directed books and websites focused on the relationship between dietary factors and endometriosis, only a small number of studies have been published on this relationship [126]. A significant association was first suggested in an animal study that observed a protective association of fish oil supplementation on surgically induced endometriosis in rabbits [127]. Human studies have since examined an array of potential dietary contributors to endometriosis risk, although replication for any given dietary factor is lacking.

Fruits and vegetables: Two studies have investigated the relationship between fruit and vegetable intake and endometriosis. An Italian hospital-based case-control study comparing 504 cases of laparoscopically confirmed endometriosis and 504 controls admitted for benign non-gynecologic conditions observed a statistically

significant inverse association of current green vegetable (OR, 0.3; 95% CI, 0.2–0.5) and fruit consumption (OR, 0.6; 95% CI, 0.4–0.8) [93]. In contrast, a more recent case-control study found increased risk associated with increased fruit consumption (OR, 1.5; 95% CI, 1.2–2.3), but no association with vegetable consumption [128].

Saturated fat and red meat: An Italian case-control study found a significantly increased likelihood of endometriosis with red meat consumption (OR, 2.0; 95% CI, 1.4–2.8) but no association with butter intake [93]. Two more recent case-control studies did not find increased endometriosis risk with increased red meat intake [111, 128]. However, one did find a marginally increased risk of endometriosis with butter intake [111]. When specifically saturated fat or animal fat was examined, no clear relationship appeared to emerge in three other studies [129–131].

Olive oil and monounsaturated fat: There is no clear relationship between olive oil, monounsaturated fats, and the risk of endometriosis. While two studies, a case-control and a cohort study, have found no association between monounsaturated fat or vegetable oil intake and endometriosis [93, 129], another case-control study focusing on benign ovarian tumors found a positive association between vegetable oil intake and endometriosis (highest vs. lowest quartiles: OR, 2.0; 95% CI, 1.2–3.2) [130].

Fish and omega-3 polyunsaturated fatty acid (PUFA): No clear association between endometriosis and fish consumption has been found in case-control studies [93, 111, 128]. In the NHSII, women in the highest quintile of long-chain omega-3 fatty acid consumption were 22% less likely to be diagnosed with endometriosis compared with those in the lowest quintile of intake (95% CI, 0.62–0.99) [129]. This inverse relation with omega-3 PUFA was also observed in a recent case-control study [131] and is supported by the findings from in vitro and animal models. Human endometrial cell survival was shown to be decreased in cultures containing a high proportion of long-chain n-3 fatty acids (i.e., eicosapentaenoic acid) [132]. In a rabbit model of surgically induced endometriosis, alpha-linolenic acid (an n-3 fatty acid) supplementation decreased concentrations of series 2 prostaglandins and endometrial implant diameter [127].

Trans fats: In the NHSII, women in the highest quintile of trans-unsaturated fat intake were 48% more likely to be diagnosed with endometriosis (95% CI, 1.17–1.88). This finding was not supported by a more recent case-control study [128]. Trans fat intake increases the circulating levels of several inflammatory markers, including IL-6 [133–135] and the markers of TNF system activation [134, 135], which are thought to be involved in the pathogenesis of endometriosis [27].

Soy/phytoestrogens: A Japanese hospital-based case-control study of 138 women undergoing laparoscopy for infertility compared 31 women with lower-stage endometriosis, 48 women with higher-stage endometriosis, and 59 controls. Higher urinary levels of the soy isoflavones genistein and daidzein were associated with decreased likelihood of higher-stage endometriosis (OR for highest vs. lowest quar-

tile 0.2 and 95% CI 0.1–0.8 for genistein and OR 0.3 and 95% CI 0.1–1.0 for daidzein), but no significant relation was observed for lower-stage disease [136]. Soy is rich in phytoestrogens which may influence circulating estrogen levels. More recently, the ENDO study found no association between urinary phytoestrogen concentrations and either surgically confirmed or self-reported endometriosis in a US population [137].

Dairy intake: The relationship between dairy intake and endometriosis is unclear. Two studies found no association between milk or cheese intake and endometriosis risk [93, 111]. A recent population-based case-control study found a nonsignificant inverse association between dairy and calcium consumption and surgically confirmed endometriosis [128]. Consistently, dairy intake was associated with a lower risk of endometriosis (OR, 0.95; 95% CI, 0.91–1.00) in the NHSII [138]. This reduction in risk appeared to be driven by an association between skim/low-fat milk and the disease.

Circulating vitamin D: Several studies have investigated the role of vitamin D in relation to endometriosis risk [139]. An Italian case-control study analyzed serum levels of 25-hydroxyvitamin-D3, 1,25 dihydroxyvitamin-D3, and calcium in 87 women with laparoscopically confirmed endometriosis and 53 controls. Levels of 25-hydroxyvitamin-D3 above the 75th percentile (>28.2 ng/ml) were associated with increased likelihood of endometriosis (OR, 4.8; 95% CI, 1.7–13.4) [140]. No significant associations were noted for 1,2,5 dihydroxyvitamin-D3 and calcium [140]. A more recent case-control study found a decreased but not significant risk of endometriosis with vitamin D levels [128]. In the NHSII, predicted plasma 25(OH)D level was inversely associated with endometriosis [138]. Women in the highest quintile of predicted vitamin D level had a 24% lower risk of endometriosis than women in the lowest quintile (rate ratio = 0.76; 95% confidence interval, 0.60–0.97) [138].

Environmental Toxins

Based on early animal studies suggesting that polychlorinated biphenyl (PCB) or dioxin exposure may influence endometriosis risk [141], there has been considerable interest in environmental exposures and endometriosis as recently summarized by Smarr et al. [142]. Environmental toxic exposures may alter risk by changing circulating hormone levels or by affecting the immune system of women exposed.

Hospital-based case-control studies conducted in various geographical regions [111, 143–148] have failed to demonstrate a consistent association between environmental toxins and endometriosis risk. These studies have varied by the types of controls included (infertile, fertile, both), the identification of endometriosis cases (self-report, laparoscopy, magnetic resonance imaging [MRI]), and the types of environmental toxins evaluated (polychlorinated dibenzo-p-dioxins [PCCDs], polychlorinated dibenzo-furans [PCDFs], dioxin-like PCBs, coplanar PCBs [cPCBs], and numerous others).

Many case-control studies examining the role of dioxins and endometriosis have observed modest but nonsignificant elevations in endometriosis risk [149–153]. Two Italian hospital-based case-control studies of nulliparous women [154, 155] examined dioxin-like and non-dioxin PCBs in relation to endometriosis risk. Both studies observed significant positive associations for nearly all the compounds they evaluated (OR for all PCBs, 6.5; 95% CI, 1.5–28.0). These studies had stricter inclusion criteria than previously reported case-control studies (women were nulliparous, age 20–40 years, without any acute or chronic disease, and with no evidence of recent weight change >10 kg). A study of infertile women undergoing laparoscopy examining a multitude of toxins (including PCDDs, PCDFs, cPCBs, PCBs, and chlorinated pesticides) revealed higher toxic equivalency (TEQ) values in controls as compared to cases (P-value = 0.02) and a nonsignificant inverse association between organochlorine exposure and endometriosis risk [156].

Two population-based retrospective cohort studies [51, 157] have examined the relationship between environmental toxins and endometriosis. The first cohort was comprised of women exposed to 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) following a 1976 chemical plant explosion in Seveso, Italy. Comparison of 19 women with endometriosis confirmed by laparoscopy or ultrasound to 277 women with no disease revealed an elevated but nonsignificant increase in the rate of endometriosis for women with TCDD levels >20 parts per trillion (ppt) (RR, 1.2; 90% CI, 0.3–4.5) [157]. The second cohort included women exposed to PCBs in 1973 through contaminated cattle feed in Michigan, USA. Of 943 women, 79 self-reported endometriosis, although medical record review confirmed only 37 cases. There was a nonsignificant but increased rate of endometriosis among women exposed to PCBs ≥4 parts per billion (ppb) compared to PCBs <1 ppb (RR,1.7; 95% CI, 1.0–3.0) [51], although both confirmed and unconfirmed cases were included in the analysis.

Small sample sizes, varying age at time of exposure, heterogeneity of control populations, differing levels of baseline exposure to toxins between geographic regions, and examination of a wide variety of toxins make a global summary statement regarding the relation between environmental toxins and endometriosis difficult. Given that true associations between environmental toxins may be of small magnitude, larger, well-controlled studies that critically consider exposure timing are required.

Endometriosis and Chronic Disease Risk

Mounting evidence suggests that women with endometriosis may also be at risk for several chronic diseases [158, 159]. While the mechanisms behind these associations are not fully understood, this research may have important implications for our understanding of disease etiology and may influence the management, care, and screening of patients with endometriosis. The current evidence supports the conclusion that women with endometriosis are at increased risk of ovarian cancer, cutaneous melanoma, and some autoimmune diseases and are at decreased risk of cervical cancer.

Cancer: At present, the data are most consistent regarding the relationship between endometriosis and risk of clear cell and endometrioid ovarian cancer. Among the 21 epidemiologic studies that investigated endometriosis in relation to ovarian cancer risk, 20 reported a positive association (including 16 reporting statistically significant findings) [160–177]. A recent pooled analysis yielded significant risk among women with endometriosis (RR, 1.46; 95% CI, 1.31–1.63) [175], findings which were also supported by a recent meta-analysis [178].

The literature is unclear about the relationship between endometriosis and breast cancer [179]. While most studies have suggested a modest positive association between endometriosis and the risk of breast cancer with effect estimates ranging from 1.08 to 3.20 (albeit only half reported statistically significant findings) [158, 160, 161, 168, 180, 181, 184, 186–188], four studies showed no clear association [169, 177, 182, 183], and five studies reported an inverse relationship [163, 166, 185, 189, 190]. However, there is significant disease heterogeneity across hormone receptors status, menopause status at time of cancer diagnosis, and molecular subtypes of breast cancer which has not been adequately addressed in the current literature [188].

The current literature on the relationship between endometriosis and endometrial and cervical cancer is sparse. For endometrial cancer, the results are inconclusive with null associations [161, 163, 168, 177], positive associations [169, 191], and inverse associations having been reported [160]. However, the results for cervical cancer are more consistent after multiple studies from Sweden seemed to show a decreased risk associated with a history of endometriosis [160, 161, 168, 169].

Among non-gynecological cancers, cutaneous melanoma has been studied most often in relation to patients' history of endometriosis. Of the 12 studies that explored this topic, seven suggested a positive association [85, 163, 166, 168, 169, 192, 193], while five studies reported no clear relation between endometriosis and cutaneous melanoma risk [86, 161, 183, 194, 195]. As presented earlier, there is growing literature on the associations between endometriosis and skin sensitivity to the sun, nevi, and freckle patterns which is consistent with possible positive association. Exploration of endometriosis in relation to other types of cancer have been sparse.

Immune system diseases: Few studies have evaluated the comorbidity of endometriosis with autoimmune diseases. A cross-sectional survey conducted among patient members of the Endometriosis Association first reported higher than expected rates of systemic lupus erythematosus, multiple sclerosis, rheumatoid arthritis, and Sjögren's syndrome than in the general female US population [34]. Some of these findings have been supported in cohort studies, but not case-control or other cross-sectional data. The most recent and largest cohort study to date, with over 37,000 endometriosis cases, found a significantly increased risk of systemic lupus erythematosus, Sjögren's syndrome, and multiple sclerosis disease in Denmark [197]. The NHSII found an association with systemic lupus (HR = 2.03; CI 1.17–3.51) and rheumatoid arthritis (HR=1.41; CI 1.05–1.89) [198]. A Spanish case-control study based on clinical record information reported no significant association between endometriosis and risk of systemic lupus erythematosus or Sjögren's syndrome [67]. Finally, no association was found between endometriosis and risk of autoimmune thyroid disorders (hypo- or hyperthyroidism) in a Brazilian cross-

sectional study [199] nor was an association found in 1400 women undergoing their first laparoscopy between endometriosis and autoimmune conditions [54].

Cardiovascular conditions: There is evidence that endometriosis may be associated with inflammation after several inflammatory markers have been shown to be elevated in women with endometriosis [200], such as intracellular adhesion molecule 1 (ICAM-1), C-reactive protein (CRP), interleukin-1 and interleukin-6 (IL-1 and IL-6), tumor necrosis factor-α (TNF-α), and vascular endothelial growth factor (VEGF). Inflammation is an important risk factor for coronary heart disease (CHD) [201, 202].

One case-control study compared intima-media thickness in women with and without endometriosis and found no significant relationship [203]. Recent data from the NHSII reported increased risk of myocardial infarction (RR, 1.52; 95% CI, 1.17–1.98), angiographically confirmed angina (RR, 1.91; 95% CI, 1.59–2.29), and coronary artery bypass graft surgery/coronary angioplasty procedure/stent (RR, 1.35; 95% CI, 1.08–1.69) associated with endometriosis [204]. Endometriosis was also associated with a higher risk of any of the three CHD events combined (RR, 1.62; 95% CI, 1.39–1.89). Part of the associations was found to be mediated through endometriosis treatments that are risk factors for CHDs, such as hysterectomy/oophorectomy and earlier age at surgery following endometriosis diagnosis.

Chronic disease risk: It is important to note that most studies investigating endometriosis and chronic diseases relied on a self-reported diagnosis of endometriosis and/or disease. Additionally, many of the studies that have assessed the associations between endometriosis and cancer to date were within population historical cohorts, where important confounding factors or mediators of the associations could not be taken into account. Given the state of the current literature, it is unclear at this time whether these relationships are causal or are being driven by shared risk factors, shared hormonal milieu, or treatments for endometriosis which alter risk.

Conclusion

Data suggests that in utero exposures, menstrual and reproductive history, anthropometrics, lifestyle, dietary, and environmental exposures may each play a role in the etiology of endometriosis. However, there are many methodological issues and limitations in current research on endometriosis. While the ideal epidemiologic study has not yet been performed, it is worthwhile to review the literature that has been presented to date (Table 1). Some authors have used Mendelian randomization methods where surrogate genetic markers that are known to be associated with an exposure of interest are used to avoid the potential biases discussed previously. Valid epidemiologic studies of endometriosis can be conducted, the results of which will aid our understanding of the pathogenesis as well as early signs and symptoms of the disease, enhancing diagnosis and perhaps even leading to disease prevention.

Table 1 A summary of risk factors for endometriosis

Risk factor	Direction and consistency of effect[a]
In utero environment	
Higher birth weight	↑, inconsistent
Maternal/paternal smoking	↓↓, inconsistent
Maternal diethylstilbestrol	↑, limited study
Menstrual and reproductive factors	
Earlier age at menarche	↑↑, consistent
Shorter menstrual cycle length	↑↑, consistent
Heavier menstrual volume	↑, limited study
Greater parity	↓↓, consistent
Lactation	↓↓, limited study
Current oral contraceptive use	↓↓, consistent
Physical characteristics	
Red hair	↑, inconsistent
Freckling	↑, inconsistent
Nevi	↑, inconsistent
Skin sensitivity	↑, inconsistent
Anthropometric factors	
Greater height	↑, inconsistent
Greater body mass index	↓, consistent
Greater childhood body mass index	↓, inconsistent
Greater waist-to-hip ratio	↓, limited study
Lifestyle factors	
Cigarette smoking	↓, inconsistent
Alcohol use	↑, inconsistent
Caffeine intake	↑, inconsistent
Regular exercise	↓, inconsistent
Night shift work	↑, limited study
Dietary factors	
Fruits and vegetables	↓, limited study
Red meat/saturated fat	↑, limited study
Vegetable oil/monounsaturated fat	- , limited study
Fish and omega-3 PUFA	↓, limited study
Trans fat	↑, limited study
Soy/phytoestrogens	↓↓, limited study
Low-fat dairy	↓, limited study
Vitamin D (25-hydroxyvitamin-D3)	-, inconsistent
Environmental factors	
PCB, dioxin exposure	↑, consistent in primates but inconsistent in women

Updated from Missmer SA, Cramer DW. Epidemiology of Endometriosis. In *Endometriosis in Clinical Practice.* DL Olive (ed), Taylor & Francis Group, London, 2004

[a]Arrows indicate the approximate magnitude of the relation: ↑, slight to moderate increase in risk; ↑↑, moderate to large increase in risk; ↓, slight to moderate decrease in risk; ↓↓, moderate to large decrease in risk; -, no association

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Peripheral Blood Biomarkers for Endometriosis

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Abstract Endometriosis is a complex disease to study and diagnose. Ultrasound can be used to diagnose cysts and endometrioma but cannot rule out peritoneal endometriosis. Endometriosis is defined as endometrium outside of uterus. It is associated with infertility and pain. If we find a diagnostic test to diagnose endometriosis in blood it would be a convenient, most probably cheap test that would discriminate women who are suffering from endometriosis from those who do not. Till today, we do not have a diagnostic test for endometriosis. For women it is a heavy psychological burden, and quick diagnosis would help reduce this burden. Studies have shown that endometriosis cost arises predominantly from productivity loss and is predicted by decreased quality of life. In this chapter we describe and discuss the current status of biomarkers (miRNAs, glycoproteins, immunological, angiogenic, cell adhesion/invasion factors) of endometriosis in peripheral blood.

Keywords Peripheral blood • Diagnosis • Endometriosis • Biomarkers • Diagnostic test • Sensitivity • Specificity • miRNA • Panel of biomarkers • Ultrasound

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Abbreviation

AUC	Area under the curve
CA-125	Cancer antigen
EDTA	Ethylenediaminetetraacetic acid
EGF	Soluble epidermal growth factor
ELISAs	Enzyme-linked immunosorbent assays
HGF	Hepatocyte growth factor
HIF-1 alpha	Hypoxia-inducible factor-1 alpha
ICAM-1	Intercellular adhesion molecule 1
IFN-γ	Interferon gamma
IL	Interleukin
miRNAs	microRNAs
MMPs	Matrix metalloproteinase
MRI	Magnetic resonance imaging
NPV	Negative predictive value
PEDF	Pigment epithelium-derived factor
PDGF	Platelet-derived growth factor
PGF or PLGF	Placental growth factor
SOPs	Standard operating procedures
TNF-α	Tumor necrosis factor
VEGF	Vascular endothelial growth factor
VEGFR1	Vascular endothelial growth factor receptor 1
VEGFR2	Vascular endothelial growth factor receptor 2
WERF	World Endometriosis Research Foundation

Introduction

Endometriosis is defined by the presence of endometrial glands and stroma outside the uterus, affecting about 10% of women of reproductive age [1] and up to 35–50% of women with chronic pelvic pain and/or infertility [1, 2]. The origin of endometriosis is still not clear; however Sampson's theory of retrograde menstruation is the most accepted theory (shed endometrial tissue flows backward through the fallopian tubes and attaches to the pelvic wall) [1, 3]. Endometriosis can be treated by surgical excision or by hormonal treatment, combined with anti-inflammatory drugs; however there is no cure for this disease [1]. The WERF EndoCost study has shown that the economic burden of endometriosis is comparable to that from diabetes mellitus and Crohn's disease [4].

The gold standard for diagnosis is laparoscopic inspection of the abdomen, preferably combined with histological confirmation of endometrial stroma and glands [5]. Nonsurgical diagnostic techniques, such as magnetic resonance imaging (MRI) and vaginal ultrasonography, can be used for the detection of endometriomas

(ovarian endometriotic cysts) but do not rule out peritoneal endometriosis or endometriosis-associated adhesions [5, 6]. A classification of the disease burden is performed using the scoring system of the American Society for Reproductive Medicine. Four stages are defined ranging from minimal to severe disease (stage I, indicating minimal disease; until stage IV, indicating severe disease) [7]. However, the classification of endometriosis according to this scoring system does not correlate with the severity of pain.

Noninvasive diagnosis of endometriosis would allow early diagnosis and treatment, with the potential to improve quality of life and reduce the societal cost related to endometriosis, and has therefore been selected as a research priority by the World Endometriosis Society (WES) and the World Endometriosis Research Foundation (WERF) [8]. The most important goal of a noninvasive diagnostic test is that no women with endometriosis are missed who might benefit from treatment [9]. At present, neither a single biomarker nor a panel of biomarkers, measurable in peripheral blood, has been validated as a noninvasive test for endometriosis [10].

The measurement of serum CA-125 levels has no value as a diagnostic tool compared to laparoscopy [5]. The lack of a noninvasive diagnostic test significantly contributes to the long delay between onset of the symptoms and definitive diagnosis of endometriosis [11].

In this chapter we describe and discuss the current status of biomarkers (miRNAs, glycoproteins, immunological, angiogenic, cell adhesion/invasion factors) of endometriosis in peripheral blood.

Diagnostic Test

Endometriosis cannot be diagnosed based on symptoms alone because of overlap with other conditions [5]. Symptoms include dysmenorrhea, dyspareunia, chronic pelvic pain, and/or infertility [12]. Clinical evaluation of the patient may reveal palpable nodules, especially during menstruation [5, 12]. Transvaginal ultrasound is only useful to detect endometriotic ovarian cysts, while endometriosis-associated adhesions, superficial peritoneal lesions, and some locations of deep infiltrating endometriosis may remain undetected [13]. Similarly, magnetic resonance imaging (MRI) is only useful to confirm the presence of endometrioma, not peritoneal lesions [14]. Laparoscopy is the current gold standard for diagnosis of endometriosis, preferably combined with histological confirmation of the lesions [5, 12]. Histological confirmation of endometriosis in a lesion biopsy requires the presence of two or more of the following features: endometrial epithelium/glands, endometrial stroma, and hemosiderin-laden macrophages [15]. However, when no biopsy is taken for histology, the difficulty to recognize lesions due to their varying appearance may cause an over- or underestimation of the extent of endometriosis [14]. Especially cases with minimal peritoneal disease, when the lesions are immediately ablated without being biopsied, may account for an overestimation of the diagnosis

[14]. Furthermore, it may be difficult to assess lesion depth, and therefore the surgeon's experience is important for the reliability of the laparoscopy data [14].

For women with a regular cycle, a partner with a normal sperm examination, lack of success in conceiving for more than 1 year, without substantial cyclic/chronic pelvic pain, and with a normal clinical examination and pelvic ultrasound, a clear indication for laparoscopy may not be present [9]. Up to 45% of these women may have endometriosis [13]. Therefore a noninvasive test will be particularly useful to discriminate between women who have endometriosis and might benefit from treatment and women who do not have endometriosis [9]. We want a test with a high sensitivity, with a low number of false negatives so that no patients with endometriosis are missed [9]. The test should not be used to screen in an asymptomatic population [13].

The delay between onset of endometriosis and eventual diagnosis may amount to an average of 6.7 years [16]. This delay may be caused by the general nature of the symptoms, their dismissal as normal, the lack of adequate imaging and clinical examination tests, and the need for a surgical diagnosis [5, 15, 17]. To reduce this delay, a blood-based diagnostic test using biomarkers is a top priority in endometriosis research [13, 18]. The implementation of such a noninvasive test in the clinic could shorten the time to diagnosis and treatment, thereby preventing disease progression, improving patients quality of life, and reducing endometriosis-associated costs [13], which may add up to €9579 per women per year [4].

Peripheral Blood Biomarkers

A biomarker is defined as "a characteristic that is objectively measured and evaluated as an indicator of normal biological processes, pathogenic processes, or pharmacologic responses to a therapeutic intervention" [19]. These biomarkers may be soluble biochemical markers, but they may also be anatomical and functional, such as physiological assessment and imaging [20]. Since clinical examination and imaging on their own only have limited use [12], the best chance to find a noninvasive diagnostic test for endometriosis may be a molecular signature. Blood is the preferred source because it is easily accessible and allows multiple measurements [21]. A review published in 2010 by May et al. summarized the realizations in the field of blood biomarkers for endometriosis [10]. Despite many efforts, no single biomarker or a panel of biomarkers has been validated as diagnostic test for endometriosis [10]. This may partly be due to inadequate control groups, low sample sizes, lack of validation, and interlaboratory differences in terms of standard operating procedures (SOPs) and enzyme-linked immunosorbent assays (ELISAs) used. The WERF has recently published a series of papers with SOP recommendations for endometriosis in order to facilitate collaborations [22, 23]. Collaborations between research centers adopting the same SOPs will allow the assembly of large sample sets necessary for biomarker research in endometriosis [13]. Despite the lack of validation, many efforts have been done to discover biomarkers for endometriosis. These will be summarized in the next paragraphs.

Immune Markers for Endometriosis

Although retrograde menstruation is a common phenomenon among women of reproductive age, not all women who have retrograde menstruation develop endometriosis [24]. An immunological/inflammatory etiology has been conjectured, as demonstrated by increased concentrations of activated macrophages, cytokines, T cells, and B cells [24]. Immune markers have also been examined extensively as possible biomarkers for endometriosis [10]. Since the comprehensive literature search of May et al. [10], only a few new studies have been investigating immune markers for endometriosis. May et al. discussed the following immune markers: interleukin (IL)-1, IL-6, and IL-8, tumor necrosis factor (TNF)-α, MCP-1, and interferon-γ (IFN-γ).

One of the several immunologic mechanisms that play a fundamental role in the immune response is the complement system. Elevated levels of C3c and C4 were reported along with an increase in SC5b-9—the membrane attack complex [25]. One study has reported lower levels of C3 and C4 in women with endometriosis, during the follicular phase [26]. Regarding biomarkers, one study has investigated the possibility of C3a, a proteolytic fragment that induces inflammatory reactions, but found no difference between women with and without endometriosis [27].

A recent review has performed a literature search on the most studied chemokines, including their receptors, which were CXCL8, CCL2, and CCL5 [28]. Chemokines represent a family of small cytokines or proteins released by cells, especially lymphocytes, and are capable of inducing chemotaxis (directed movement through the chemicals of the microenvironment) in nearby responsive cells, directing the cellular migration [28]. Researchers have reported significantly higher levels of CXCL8 in patients with endometriomas versus controls [29–31], whereas other studies reported no statistically significant difference of this marker among endometriosis subjects and controls [32, 33].

No difference in the serum levels of the inflammatory markers CD40, CD40L, and a disintegrin and metalloproteinase domain 8 (ADAM8) was detected between endometriosis patients ($n = 47$) and controls ($n = 29$) [34]. Additionally, interleukins 10, 12, 17, and 23 levels were comparable between infertile controls and endometriosis patients with infertility [35].

Recently, IL-8, MCP-1, and RANTES showed potential as a biomarker, being significantly increased in endometriosis cases versus controls, respectively, 46.1, 50, and 75% of reported studies [28]. Mihalyi et al. reported a panel consisting of luteal plasma levels of IL-8, TNF-α, and CA-125 that was able to distinguish between 201 women with endometriosis and 93 controls with a normal pelvis with a sensitivity of 89.7% and a specificity of 71.1% [29]. In a study by the same group, univariate analysis showed the differential expression of several cytokines and chemokines in 232 women with endometriosis and 121 controls [30]. Surprisingly no cytokines or chemokines were included in the final proposed panel of biomarkers after multivariate analysis [30]. Whether cytokines are suitable to discriminate endometriosis patients from patients with other pelvic pathology is still questionable [30] since some studies included have low power due to small sample size and study designs vary in the assessment criteria for the markers, the state of the patients (e.g., phase of the cycle and stage of disease), and the nature of the controls [28].

Glycoproteins

Glycoproteins are important regulators of a number of biological functions, such as immune recognition, cell signaling, proliferation, and differentiation [36]. A number of glycoproteins has been investigated for their use as biomarkers for endometriosis [10, 37].

Cancer antigen (CA)-125 is until now the most investigated biomarker for endometriosis and has been shown to be elevated. This has been reviewed by Mol et al. and May et al. [10, 38]. Its specificity for endometriosis is quite low, as it is also a marker for non-endometriotic ovarian cysts, myomas, and for several cancers, such as ovarian cancer, endometrial cancer, cervix cancer, and lung cancer [39]. Further, the sensitivity to detect all stages of endometriosis is low, although it is better for stage III–IV endometriosis [38]. CA-125 has been measured alone or in combination with some other markers but without proven diagnostic value [40–45]. Some panels were promising, but results remain to be validated [29, 30, 46–48]. Additionally, CA-125 may be a good marker to monitor the effects of medical treatment [49, 50].

The ideal cutoff of CA-125 has been debated [45]. For epithelial ovarian cancer, a cutoff of 35 IU/mL was set [51], which has largely been adopted by the endometriosis field [38, 52]. Kitawaki et al. suggested that for women without endometrioma the combination of two cutoffs (20 and 30 IU/mL) may be preferable over only one cutoff to reach maximum specificity and sensitivity [52]. Xavier et al. proposed a value of 22.6 IU/mL as optimal cutoff [53].

CA-19-9, another ovarian tumor marker, has been shown to be elevated in endometriosis and may be correlated with disease severity [10, 54] and has a comparable or lower sensitivity than CA-125 for the detection of endometriosis [10, 54]. Further CA-15-3 and CA-72 have shown mostly contrasting results [37].

Glycodelin A has been shown to be upregulated in patients with endometriotic ovarian cysts [55] and in another study in patients with stage III–IV endometriosis [56]. Furthermore, it was part of a panel predicting ultrasound-negative endometriosis with good accuracy together with CA-125, VEGF, and annexin V [30]. Glycodelin A has been shown to inhibit sperm-egg binding and block the innate immune response [56].

Some other glycoproteins have been suggested to be increased in patients with endometriosis, such as osteopontin [57, 58], fetuin A [59], Zn-alpha2-glycoprotein [11], and Gremlin-1 (only in the follicular phase) [60]. Pigment epithelium-derived factor (PEDF) levels were decreased in serum of women with endometriosis [61]. Serum follistatin levels could differentiate between patients with endometrioma and patients with other benign ovarian cysts [62]; however this was not confirmed by another study [63]. An increase in haptoglobin β chain isoforms has been shown in women with endometriosis [64]. However, two independent studies using a proteomics approach reported a downregulation of haptoglobin in women with endometriosis [65, 66].

Cell Adhesion and Invasion

Cell adhesion and invasion are important steps in the establishment of endometriosis [67]. Cell adhesion markers may regulate cell–cell interactions, and their abnormal expression by endometrial cells has been documented for endometriosis [10, 67]. The most investigated cell adhesion marker is intercellular adhesion molecule 1 (ICAM-1) [10]. Conflicting results exist on the expression of ICAM-1 in endometriosis [10]. ICAM-1 levels may rise during stage I–II and decrease in stage III–IV [10]. ICAM-1 has been included in the biomarker panel to predict ultrasound-negative endometriosis together with CA-125, VEGF, and annexin V [30].

Remodeling of the extracellular matrix by matrix metalloproteinase is thought to be an important step in the development of endometriosis [67]. Therefore, an increase in MMPs is expected in endometriosis, which has been reported in blood for MMP-2 [68], MMP-9 [69], and mRNA levels of MMP-3 [46, 70].

Angiogenesis

Studies have shown that angiogenesis is involved in the pathogenesis of endometriosis. Vascular endothelial growth factor (VEGF), vascular endothelial growth factor receptor 1 (VEGFR1), vascular endothelial growth factor receptor 2 (VEGFR2), placental growth factor (PGF or PLGF), and hypoxia-inducible factor-1 alpha (HIF-1 alpha) are part of the biological system which plays a key role during angiogenesis [71–79]. A few controversial data exist regarding the use of angiogenic factors as peripheral blood biomarkers in endometriosis. The most studied proangiogenic factor in endometriosis is VEGF. Peripheral blood VEGF levels have been reported to be either increased [80–83] or to be similar [32, 84, 85] in women with endometriosis when compared to controls, probably due to differences in study design and methodology.

A recent study described a panel of biomarkers including VEGF in two panels to detect minimal/mild endometriosis with 80% sensitivity [30]. Interestingly, another study followed up patients with advanced endometriosis and showed a reduced VEGF-A levels after laparoscopic excision of the lesions [82, 86]. No difference was found between endometriosis patients and control women when soluble epidermal growth factor (EGF) and platelet-derived growth factor (PDGF) were investigated [10]. In contrast, hepatocyte growth factor (HGF) was suggested to be elevated in women with endometriosis [87], although this was not confirmed in another study [10, 88].

Omics

miRNA in Endometriosis

In addition to genetic mechanisms that may be at the basis of endometriosis, epigenetic mechanisms, such as microRNAs (miRNAs), have been investigated [89]. miRNAs are short single-stranded noncoding RNAs of ~21 nucleotides which post-transcriptionally regulate gene expression of several mRNAs [90]. They control many mechanisms in the body, including some which are important in the development of endometriosis, such as inflammation, cell invasion, and angiogenesis [91]. Furthermore, miRNAs have been implicated in the pathogenesis of endometriosis [90]. In cancer, the miRNA profiles in blood correlated well with the cancer tissue, which implies that miRNAs are secreted from the tissue into the circulation [92]. Use of miRNA signatures has been proposed for diagnosis of various diseases, such as cancer, neurological disorders, and cardiovascular and rheumatic diseases [93]. miRNA differences between eutopic and ectopic endometrium have been observed in endometriosis [90, 94]. miRNAs can be secreted into the blood where they are transported in microvesicles, which protect them from RNases, making them very stable and potentially good biomarkers [95]. In peripheral blood, miRNA has been investigated as potential biomarkers for endometriosis [92, 96, 97]. Jia et al. showed that the combination of three downregulated miRNAs in plasma (miR-17-5p, miR-20a, and miR-22) resulted in an area under the curve (AUC) of 0.90 [92]. Suryawanshi et al. showed that three plasma miRNAs (miR-16, miR-191, miR-195) were upregulated in endometriosis and had an AUC of 0.90 to differentiate between healthy and endometriosis cases [97]. Wang et al. investigated the miRNA values in serum samples, and after discriminant analysis, the combination of miR-199a, miR-122, miR-145*, and miR-542-3p proved to be the best to predict endometriosis, with an AUC of 0.994 [96].

Some of the different findings between the studies may be explained due to methodological reasons. Plasma may show higher miRNA concentration than serum [98], although other studies showed no differences [97, 99], or an increased concentration in serum [100]. These discrepancies could be attributed to preanalytical variability in blood tube type and SOPs [98]. Some investigators prefer plasma because of possible interference of released miRNAs during the coagulation process in serum [100]. Plasma can be collected in ethylenediaminetetraacetic acid (EDTA) tubes [92] or heparinized [97] tubes, which may also have an influence as heparin has been suggested to inhibit the RT-PCR reaction, although it may only be a minimal effect [97]. Another methodological issue is the lack of consensus on the normalization control to determine the relative expression of the miRNAs. In the three studies currently published on miRNAs in blood concerning endometriosis, three different normalization controls were used, namely, miR-16 [92], miR-132 [97], and U6 [96].

Furthermore, miRNAs have been suggested to be hormonally regulated [101]. Therefore, menstrual cycle and the use of hormonal medication may be an issue.

However, it has been suggested that cycle-dependent changes in miRNA levels are rather present on a local level and are not reflected systemically [95].

More research on the discovery of miRNAs in peripheral blood of endometriosis patients is necessary to get a better image of which miRNAs may be potential biomarkers in a diagnostic test for endometriosis.

Metabolites

To complete the picture of endometriosis besides studying mRNA, miRNA, and proteins or peptides, maybe the changes in metabolites can give rise to a diagnostic test for endometriosis. One study has observed that the menstrual cycle has an influence on the metabolic changes in plasma of premenopausal women [102]. In endometriosis patients (n = 64), stearic acid was significantly reduced compared with controls (n = 74) (p = 0.030) [103]. Another study found that eight metabolites and 81 metabolite ratios were significantly higher in the endometriosis group compared to controls (n = 52) [104]. The combination of hydroxysphingomyelin C16:1 and the ratio between phosphatidylcholine C36:2 to ether-phospholipid C34:2 provided a sensitivity of 90.0% and a specificity of 84.3% for the detection of endometriosis [104]. Higher levels of lactate, 3-hydroxybutyrate, L-alanine, glycerophosphatidylcholine, L-valine, L-leucine, L-threonine, 2-hydroxybutyrate, L-lysine, and succinic acid were found in patients suffering of endometriosis, whereas glucose, L-isoleucine, and L-arginine were decreased [105]. Future studies are necessary to elucidate whether metabolites could be integrated in a noninvasive diagnosis test for endometriosis.

Proteomics

Proteomics and endometriosis in peripheral blood will be described and discussed in detail in the following Chap. 10.

Clinical Factors Integrated in Diagnostic Test

Endometriosis cannot be diagnosed based on symptomatology or clinical tests alone [12]. Symptoms include infertility/subfertility, chronic pelvic pain and pain during menstruation, defecation, and/or sexual intercourse [12, 106]. Short menstrual cycles, heavy menstrual bleeding [107, 108], and a pinpoint cervix [109] have been related to an increase in endometriosis risk. Endometriosis may be suspected after finding a fixed uterus, adnexal masses, or palpable nodules during a clinical examination [5, 12].

A study by Eskenazi et al. showed that endometrioma could be predicted reliably using a classification tree that incorporated ultrasound, physical exam, dysmenorrhea, and dyspareunia [110]. Nnoaham et al. published a paper in 2012 about a patient-reported questionnaire, stating that stage III–IV endometriosis could be predicted by a history of benign ovarian cysts and menstrual dyschezia with good sensitivity and specificity; however performance to detect any-stage endometriosis was poor [111].

The chance that these clinical factors can predict the presence or absence of endometriosis on their own is minimal, except possibly for patients with endometrioma. However, the clinical factors may provide an added value when they are incorporated in a diagnostic test for endometriosis in combination with molecular markers.

Future Trends

Overall, most endometriosis biomarker studies have remained at the level of Phase I [10] and only a few have made it to Phase II studies. Clearly, there is a need for well-designed Phase II and Phase III trials to make progress in this field [112].

Development of a diagnostic test for diseases is in general a long procedure. Since endometriosis a complex disease and the etiology and pathogenesis is still not known precisely, it will be difficult to find a diagnostic test with a high sensitivity and specificity. Animal models such as the baboon endometriosis model would be ideal to study markers before and after endometriosis. In the study design of future studies, biostatisticians should always be involved in order to transfer comprehensive biomarker knowledge into diagnostic test [13]. Additionally, a combination of clinical factors and analytes in a diagnostic test might give rise to a higher sensitivity and specificity test for endometriosis. Collaborations are necessary to test how useful the proposed diagnostic test is. Furthermore for successful collaboration, it is important to have standard operating procedures regarding collection, storage, and data analysis [23].

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Biomarkers for Endometriosis in Saliva, Urine, and Peritoneal Fluid

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Abstract Endometriosis is a pain syndrome which affects 35–50% of women with chronic pelvic pain and/or infertility. It is frequently misdiagnosed leading to delays in patients obtaining appropriate treatments. The most accurate currently available mode of diagnosis is through laparoscopy with histological confirmation. In this chapter we describe noninvasive and semi-invasive modalities to obtain biologic biomarkers which may be an adequate screening tool for patients with endometriosis who are symptomatic with normal transvaginal ultrasounds, whom are at highest risk for diagnosis delay. In this chapter we will review noninvasive (urinary, salivary) and semi-invasive biomarkers (peritoneal), as screening and diagnostic methodologies for women symptomatic endometriosis and normal ultrasound findings. Few genetic markers have been identified through DNA amplification of buccal swabs as well as hormonal markers, and this is an area with lots of potential. Various urinary peptides and proteins are discussed, some with more potential than others, which require studies with larger sample sizes, and need to be studied in more diverse populations. We also review the extensively studied peritoneal biomarkers which include cytokines, immune modulators, and growth factors. Though many of the biomarkers described have a lot of potential, there is not one that stands above the rest. Validation of these studies in larger sample sizes including various study populations is required prior to their applicability into the clinical setting. It is most probable that the answer lies in the study of combination of biomarkers and the identification of ideal panel that can predict the diagnosis and the severity of endometriosis.

Keywords Endometriosis • Biologic markers • Saliva • Urine • Peritoneum • Hormonal • Genetic • Cytokines • Immune modulators

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Introduction

Endometriosis is a pain syndrome that occurs in 6–10 % of reproductive-age women overall and affects 35–50% of women with chronic pelvic pain and/or infertility [1–3]. Because of the many different potential presenting symptoms, it can go misdiagnosed for many years by many different health care providers including gynecologists. In a survey taken by women with endometriosis, there was a 65% misdiagnosis rate, and 46% of the 7025 women had to see five doctors or more before obtaining the correct diagnosis [4, 5]. On average, women with endometriosis can suffer an approximate 8-year delay before obtaining their diagnosis [6].

Given the estimated delay in obtaining the diagnosis and therefore delay in the appropriated treatment of endometriosis, the health care costs to patients and society are excessive. In [7], Simoens et al. determined that the cost of evaluating and treating endometriosis is comparable to other chronic diseases such as diabetes, Crohn's disease, and rheumatoid arthritis. This study estimated that the yearly cost to the Unites States was 49.6 billion Euros (61.8 billion dollars), mostly due to surgery (29%), monitoring tests (19%), hospitalization (18%), and physician visits (16%). The goal with early and accurate diagnosis is to obtain appropriate treatment sooner, improvement in quality of life, and decrease indirect and direct cost to the patient and society as a whole.

Currently, the tools we have to detect endometriosis are either invasive or inaccurate, with low specificity and sensitivity. Pelvic ultrasound is useful in the diagnosis of ovarian endometriosis cysts [8, 9] but may not reliably identify peritoneal endometriosis or endometriosis-associated adhesions, with little ability in diagnosing deep infiltrating endometriosis (DIE) [10]. It is also important to note that the rate of detection of endometriosis via ultrasound is dependent on the experience of the sonographic examiner [11, 12]. MRI may be useful in screening for upper intestinal tract involvement if one has a high suspicion with a normal transvaginal ultrasound. However, if there is a strong clinical suspicion of intestinal involvement, yet the transvaginal sonography shows no intestinal infiltration, MRI could be useful to exclude lesions of the upper intestinal tract [11, 13–15]. In the current standard of care, the definitive diagnosis of endometriosis involves a diagnostic laparoscopy with concurrent histological findings. Laparoscopy is an invasive approach which can be associated with some morbidity and may not be beneficial or necessary in treatment of minimal to mild endometriosis [16]. It is imperative that specific and accurate markers of endometriosis be identified, in order to avoid an unnecessary surgical intervention and provide adequate medical treatment.

We will review noninvasive methods of diagnosing endometriosis with the use of salivary and urinary markers, as well as by semi-invasive identification of biomarkers from the peritoneal fluid. The ideal screening test should be easily accessible, affordable, reliable, accurate, and least invasive as possible (WHO). It should make it possible for an early diagnosis of endometriosis to be obtained in symptomatic

patients. These noninvasive and minimally invasive biomarkers could also be used to monitor disease recurrence and/or response to medical and surgical therapies. Our ability to identify reliable and reproducible biomarkers of endometriosis will change our approach to the diagnosis and management of endometriosis. These screening modalities would potentially most benefit women with pelvic pain and/or subfertility with otherwise normal pelvic ultrasounds. These women are at highest risk for diagnosis delay, therefore resulting in treatment delay. The hope is to be able to identify a biomarker or a group of biomarkers with high sensitivity and specificity for the diagnosis of endometriosis is rarely missed.

In this chapter we will explore urinary peptides and proteins and genetic markers through DNA amplification of salivary swabs, as well as the multitude of cytokines and growth factors that have been identified in the peritoneal fluid to be associated with endometriosis. All the biomarkers that we know exist have a lot of promise but are not yet conclusive, reproducible, accurate, or highly sensitive.

Salivary Markers of Endometriosis

One of the most convenient sources of testing for disease markers is through saliva. A salivary swab would allow for quick noninvasive collection of secretions and cells. From there, secreted factors such as hormones and specific genetic modifications can be quickly identified, analyzed, and used to assess the absence or presence of disease. Salivary markers have been extensively studied as diagnostic tools in diabetes and Cushing syndrome, and the potential exists to develop salivary markers for endometriosis [17]. Proper sample collection is essential to ensure the quality of the intended biomarker and to prevent reduction in the quantity. Guidelines for this were recently outlined by the World Endometriosis Research Foundation Endometriosis Phenome and Biobanking Harmonization project (WERF-EPHECT). These guidelines emphasize the need to use validated collection tools which ensure high DNA quality, such as the Oragene and DNAgard kits, time collection relative to daily biomarker fluctuation, and to ensure patients have not consumed or were exposed to any substance which can compromise specimen quality. They also elaborate on the need to store the specimens in a chilled environment or at −80 °C for long-term storage. Each of the aforementioned studies, at least in part, meets these guidelines for sample collection and storage.

Salivary markers for endometriosis can be classified into genetic markers (i.e., DNA or RNA based) and nongenetic markers. As is described below, the majority of studies involve nongenetic markers, e.g., proteins and steroids. There is a paucity of genetic markers studied in the saliva; the vast majority of genetic markers have been studied in serum. However, numerous microarray studies have shown that saliva is highly comparable to serum in finding distinct biomarkers over thousands of genetic variants.

Hormonal Markers

A handful of studies, starting in the 1990s, sought to determine if non-nucleic acid-based salivary markers can be identified in patients with endometriosis. One study looked at follicular and luteal phase salivary progesterone in women with endometriosis and infertility and found an increase in salivary progesterone in 45% of the ovulatory cycles. However, this study only involved 23 women and lacked a control, and the number of cycles measured was inconsistent among all of the subjects [18]. Another putative salivary marker studied was cortisol; a recent cohort study looked at morning salivary cortisol and found significant morning hypocortisolism in women with endometriosis. Unfortunately, this data was not correlated with ASRM endometriosis staging criteria or disease duration [19]. Nevertheless, the decreased morning cortisol could be a putative marker, especially if combined with genetic indicators of endometriosis. Little work has been done on other markers such as salivary estrogen and CA-125. Altered levels of both markers have been seen in studies looking at non-salivary markers, and this could be an additional line of future research.

Genetic Markers

Increasing evidence has shown a polygenetic basis for endometriosis [20]. Twin studies have shown an inheritable basis for endometriosis with a five- to sevenfold increased incidence among first-degree relatives [21]. This forms the basis for the search for practical and reliable genetic markers that can be used for diagnosis, prognosis, or both.

Many genetic markers have been considered including polymorphisms in members of the cytochrome p450 family; progesterone receptor including PROGIN; various key genes involved in immune cell function such as FOXP3, FCRL3, and NF-kB among others; CDKN2BAS RNA; and the V109G variant of the p27 gene. The vast majority of these genetic variants have been identified using serum samples. This paucity of studies looking at genetic variants in saliva could be due to the difficulty presented with sorting out human DNA from normal salivary flora. However, since the cells present in the oral cavity should have the same genetic variations present in other tissues (excluding any neoplastic tissue), then serum genetic markers could be considered worthwhile salivary markers. This begs the question of whether salivary DNA biomarkers are comparable to those found in serum. Several studies have shown this including one studying the performance of serum and salivary samples that were compared on a microarray platform testing for numerous drug metabolism single-nucleotide polymorphisms. This study showed a 98.7% concordance between serum and saliva samples when at least 31.3% of the DNA was recoverable [22].

To determine which genetic variants may be implicated in endometriosis, various biochemical and bioinformatics methods are used. These methods include analysis of restriction fragment length polymorphisms combined with first- and second-generation sequencing data and genome-wide association study (GWAS) data.

Restriction fragment length polymorphism (RFLP) analysis is based on the idea that if a patient has a genetic mutation at a specific site, then this could influence the cleavage ability of restriction enzymes. These enzymes can only cleave a piece of DNA if precise sequences of DNA bases are present. This altered cleavage pattern is then detected using DNA electrophoresis, and the exact sequence change can be pinpointed using first-generation (Sanger) sequencing methods. An alternative is to use next-generation sequencing (454, Illumina, etc.) which, although more expensive, can be used to read much larger tracts of human DNA in a quicker fashion. Bioinformatics work can be used to detect significant single-nucleotide polymorphisms based upon the frequency of significant changes in a certain gene's DNA sequence.

Although these techniques have been used to discover a wide variety of genetic variants, numerous shortcomings exist. RFLP analysis, although relatively inexpensive and quick, can oversimplify a group of polymorphisms, especially if the subsequent fragments have exactly the same number of nucleotides. This then could lead to grouping together of two or more variants as one, thereby inaccurately describing the overall diversity of genetic variants. GWAS studies also have numerous shortcomings in that many of the variants found do not have any clear clinical or biochemical significance, since they tend to occur in noncoding areas of the genome. Furthermore, the loci found in GWAS studies tend to have weak cumulative predictive power for specific phenotypes [23]. Nevertheless, these techniques have yielded distinct genetic targets and therefore form the basis in the search for the genetic variants associated with endometriosis.

Cell-Cycle Regulatory Factors

Research into putative genetic markers for endometriosis has focused mainly on factors affecting progression through the cell cycle. One such potential target is cyclin-dependent kinase inhibitor 2B antisense RNA (*CDKN2BAS*). In a GWAS study from a group from Utah where only salivary samples were studied, it also showed a significant association with WNT4 in a cohort of 2019 patients with laparoscopically confirmed endometriosis and 5292 controls [24]. These results were later confirmed in a case control study and meta-analysis from a group in Italy with 305 patients with endometriosis and 2710 controls. In addition the group found WNT4 and FN1 as additional loci for which polymorphisms could be indicative of endometriosis [25]. However, the above studies were only done using serum samples. Using only salivary samples, a GWAS study from a group from Utah showed another significant association with WNT4 and endometriosis in a cohort of 2019 patients with laparoscopically confirmed endometriosis [26].

Variations in *p27* have recently arisen as a new potential target for endometriosis. *p27* is also known as cyclin-dependent kinase inhibitor 1B. It is an enzyme inhibitor that is encoded by the *CDKN1B* gene. This protein binds to and prevents activation of cyclin E-CDK2 and cyclin D-CDK4 complexes which can arrest the cell cycle at the G1 level. A study from Brazil using buccal swabs showed a significant prevalence of a V109G substitution in the *p27* gene of women with endometriosis [27]. Thus far the V109G variant of *p27* is the most promising candidate as a salivary marker of endometriosis.

Summary

In retrospect, there are many proposed but few worthwhile targets for genetic testing and fewer still which have been identified in saliva. Additional microarray studies and our improved mechanistic understanding of endometriosis have since led to new genetic targets being suggested as viable diagnostic markers [28]. As shown among a myriad of genes, genetic variants can be found in both serum and salivary samples with high comparability. Future research should therefore focus on confirming the presence of genetic variants in saliva that were previously found in serum samples. From this initial proof-of-principle work, more investigation would need to be done with larger, more varied patient populations to identify a reliable salivary marker.

Urinary Biomarkers of Endometriosis

In an effort to find a quick and easy method to identify markers for women with endometriosis, urine is an optimal specimen as it can be obtained in abundant quantities with no adverse effects. Urine is specifically advantageous for the identification of proteins associated with endometriosis, as it may contain a simpler dynamic range and the highest concentration of plasma proteins in body fluids due to renal filtration [29]. It is for these reasons that urine is well suited to proteomics or the study of proteins. Factors that may alter the concentration and expression of urinary proteins, including dehydration, medications such as diuretics, and other disease processes, are a conceivable weakness of urinary proteomics.

Proteomic techniques used to identify and study proteins in the urine include two-dimensional electrophoresis (2-DE), Western blot, enzyme-linked immunosorbent assay (ELISA), matrix-assisted laser desorption/ionization time of flight (MALDI-TOF), mass spectrometry (MS), liquid chromatography (LC), and liquid chromatography tandem mass spectrometry (LQ-MS/MS). Unfortunately MALDI-TOF by itself does not elucidate protein identities, which are necessary for validation studies and its application in the clinical setting. However this technique may be combined with tandem mass spectrometry, which does successfully identify

proteins [30]. In the past 7 years, six studies have emerged evaluating urinary biomarkers of endometriosis using the above techniques. The significant urinary biomarkers found in the literature to date and their sensitivity and specificity for endometriosis where applicable are listed in Table 1. All of these studies meet the required minimum standard operating procedures detailed by the WERF EPHect global consensus guidelines. These studies thus offer a minimization in the variability between different study and center biospecimen results and therefore facilitate intercomparisons.

Soluble fms-Like Tyrosine Kinase

With the use of ELISA in 2007, Cho et al. identified an anti-angiogenic factor, soluble fms-like tyrosine kinase (sFlt-1), whose urinary levels when corrected for creatinine were significantly higher in patients with documented and confirmed endometriosis compared to controls [31]. This study also found a correlation of urinary sFlt-1 expression corrected for creatinine with the severity of endometriosis disease, in that its levels were significantly higher in patients with minimal-to-mild endometriosis (stages I and II), but the levels in moderate to severe disease (stages III and IV) did not show significant differences. Also of interest, levels of urinary sFlt-1 corrected for creatinine were significantly increased in endometriosis patients compared to controls during the secretory phase of their menstrual cycle, but its levels did not vary throughout the menstrual cycle in the control group. While significant increases in sFlt-1 levels corrected for creatinine were discovered in the urine of patients with endometriosis compared to controls, this study found no difference in the serum levels of sFlt-1 between these groups.

The complicated process of angiogenesis is essential to endometriosis lesions successfully establishing their own blood supply at ectopic locations in the body. Soluble fms-like tyrosine kinase downregulates vascular endothelial growth factor (VEGF), and perhaps this downregulation of VEGF-mediated angiogenesis is part of the pathology of endometriosis. The overexpression of sFlt-1 and its contribution to pathologic neovascularization in breast and colon cancers have been previously reported [37]. It is of interest that sFlt-1 was significantly higher in stage I and stage II endometriosis, suggesting its expression and angiogenic mechanisms in endometriosis may alter with progressing disease.

Matrix Metalloproteinases

Using zymography, which is an electrophoretic technique to detect proteases, Becker et al. in 2010 identified three matrix metalloproteinases (MMPs), MMP-2, MMP-9, and MMP-9/NGAL (neutrophil gelatinase-associated lipocalin), whose urinary levels were significantly higher in patients with endometriosis compared

Table 1 The primary outcomes of studies on urinary biomarkers of endometriosis

Urinary biomarker	Cases	Controls	Primary outcome		Study
Soluble fms-like tyrosine kinase	*N* = 46 Confirmed endometriosis: Stage I = 10 Stage II = 5 Stage III = 8 Stage IV = 23	*N* = 24 Normal pelvis = 4 Dermoid cyst = 8 Benign ovarian cyst = 8 Hydrosalpinx = 2 Tubal reversal = 2	sFlt-1 (pg/mg Cr)		Cho et al. [31]
			Case	Control	
			0.54 ± 0.09	0.26 ± 0.06	
Matrix metalloproteinases (MMP)	*N* = 33 Confirmed endometriosis	*N* = 24 No evidence of disease		Odds ratio of having disease	Becker et al. [32]
			MMP-9/NGAL	6.3	
			MMP-9	7.8	
			MMP-2	4.8	
			Any MMP above	8.3	
Vitamin D-binding protein	*N* = 57 Confirmed endometriosis Stage I/II = 15 Stage III/IV = 52	*N* = 38 Dermoid cyst = 19 Benign ovarian cyst = 20	Endometriosis diagnosis		Cho et al. [33]
			Sensitivity	Specificity	
			58%	76%	
Cytokeratin-19	*N* = 11 Confirmed endometriosis	*N* = 6 No evidence of disease	Case	Control	Tokushige et al. [34]
			Present	Absent	
Peptide panels	*N* = 23 Stage I/II = 10 Stage III/IV = 13	*N* = 16 No evidence of disease	Endometriosis diagnosis		El-Kasti et al. [35]
			Sensitivity	Specificity	
			75–82%	71–88%	
Peptide panel	*N* = 60 Stage I/II = 26 Stage III/IV = 34	*N* = 62 No evidence of disease	Endometriosis diagnosis		Wang et al. [36]
			Sensitivity	Specificity	
			90.9%	92.9%	

with controls [32]. Matrix metalloproteinases are endopeptidases that degrade and remodel the extracellular matrix of cells and enable migration and invasion of these cells into tissue [38]. It makes sense that MMPs may be involved with the invasive nature of endometriosis, and indeed MMP-2 and MMP-9 are expressed in both eutopic and ectopic endometrial tissue in women with endometriosis [39, 40]. In the study by Becker et al., the odds ratio of MMP-2 level elevation in the urine and having endometriosis was 4.8. For MMP-9/NGAL this odds ratio was 6.3, and for MMP-9 the odds ratio was 7.8 [32]. MMP-9 was therefore identified as the single best predictor of endometriosis in this set of MMPs. Longitudinal data was also collected using these MMP biomarkers, and the presence or absence of later symptoms and/or disease correlated with MMP urinary levels. While MMP-2, MMP-9, and MMP-9/NGAL were found to be significantly elevated in the urine of patients with endometriosis compared to controls in the aforementioned study, a later study on the serum levels of matrix metalloproteinases by Melvezzi et al. in 2013 [41] found only MMP-2 levels to be elevated in the setting of stage III or IV disease compared to controls and highlights the lack of correlation between biomarker levels found in the urine and the blood.

Vitamin D-Binding Protein

A study by Cho et al. in 2012 used 2-DE, Western blot, ELISA, and LC-MS/MS to identify five urinary proteins that were most elevated in patients with endometriosis compared to controls. These proteins included prealbumin, enolase-I, alpha-I antitrypsin, chain A solution structure of Bb domains of human protein disulfide isomerase, and vitamin D-binding protein (VDBP), of which VDBP was the highest expressed. Vitamin D-binding protein is a factor involved with chemotaxis and facilitates recruitment of monocytes, neutrophils, and fibroblasts. It also can be converted by B and T cells into a macrophage-activating factor [42, 43]. Its elevated levels in the urine of patients with endometriosis may be connected to the subclinical, systemic inflammatory process found in endometriosis. In this study, vitamin D-binding protein levels when corrected for creatinine (VDBP-Cr) were significantly higher in patients with endometriosis compared to the control group [33]. No significant correlations were noted between VDBP-Cr levels and disease severity. Also unfortunately, VDBP-Cr showed only 58% sensitivity and 76% specificity for the diagnosis of endometriosis. Analysis of VDBP-Cr when combined with serum CA-125 levels showed improved values with 73% sensitivity and 97% specificity; however even so this test's value as a surrogate diagnostic marker of endometriosis may be limited [33]. While VDBP was discovered in this study to be elevated in the urine of women with endometriosis compared to controls, Borkowski et al. found that VDBP concentrations in the serum are not affected in women with endometriosis. This again illustrates that the concentration and usefulness of biomarkers of endometriosis may be disparate between categories of biospecimens [44].

Cytokeratin-19

Two-dimensional electrophoresis and MALDI-TOF-MS were used by Tokushige et al. in 2011 to identify that cytokeratin-19 (CK-19) is present in the urine of women with endometriosis but not detectable in the urine of controls [34]. No correlation was found between the severity of disease and the amount of CK-19 detected in the urine of patients with endometriosis. However, in a recent study by Kueseel et al. in 2014 [45], these results could not be verified. This latter study also compared the CK-19 levels in serum between patients with endometriosis and controls and found no difference. Cytokeratin-19 is a cell structural protein and is expressed in epithelial cells and many cancers [46]. Cytokeratin-19 expression has been found in endometrial tissue of women both with and without endometriosis [47]. Not much is known regarding the pathophysiology of CK-19's role in endometriosis, and more study is required.

Peptide Panels

While the above studies identified proteins elevated in the urine of women with endometriosis, and some of them evaluated an individual protein's diagnostic value, El-Kasti et al. and Wang et al. in 2011 and 2014, respectively, evaluated the efficacy of diagnosis using panels of peptides. Via urinary analysis with MALDI-TOF-MS, El-Kasti et al. identified six peptides significantly different in endometriosis patients with moderate to severe disease (stages III and IV) compared to controls with the absence of disease. The masses of these peptide profile peaks were found at 1519.3, 1767.1, 2660.88, 9767.6, 1824.3, and 3265.4 Daltons (Da). Additionally, seven peptides were identified to be significantly different in patients with minimal to mild disease (stages I and II) compared to patients with moderate to severe disease (stages III and IV), with profile mass peaks at 2984.3, 3280.9, 1933.8, 2641.5, 2660.0, 2767.4, and 6157.3 Da. The levels of these peptides in both analyses correlated with the menstrual cycle. Comparing moderate/severe disease to controls, four peptides were significantly different during the periovulatory phase (mass peaks at 1519.3, 1767.1, 2660.88, and 9767.6 Da) and two during the luteal phase (1824.3 and 3265.4 Da). Comparing minimal/mild to moderate/severe disease, two peptides were significantly different during the periovulatory phase (mass peaks at 2984.3 and 3280.9) and five during the luteal phase (1933.8, 2641.5, 2660.0, 2767.4, and 6157.3 Da.). In the comparison of moderate/severe disease to controls, the periovulatory peptide mass 1767.1 Da and the luteal peptide mass 1824.3 Da both showed sensitivities of 75% and 85% and 71% specificity, respectively. In the comparison of minimal/mild disease to moderate/severe disease, the periovulatory peptide mass of 3280.9 Da and the luteal peptide mass of 1933.8 Da had sensitivities of 82% and 75% and specificities of 88% and 75%, respectively [35]. This study indicates that peptide panels may be used in the future as surrogate biomarkers for endometriosis disease progression.

Similarly using MALDI-TOF and LC-MS/MS technology, Wang et al. identified and created an algorithm of five peptides (m/z = 1433.9, 1599.4, 2085.6, 3217.2, and 6798.0) with significantly higher levels in 60 patients with endometriosis compared to 60 controls to use as a diagnostic tool. When used in a blind test, this model had sensitivity of 90.9% and specificity of 92.9% to predict endometriosis prior to laparoscopy. In contrast to the previous study, no significant differences were noted in peptide levels throughout the menstrual cycle [36].

It is exciting that urinary peptide and protein profiles may be able to determine the presence and severity of endometriosis and may be used in the future for both its diagnosis and potentially for monitoring therapeutic efficacy. The high concentration of proteins within urine compared to other fluid biospecimens makes it an advantageous medium for proteomics and explains the lack of correlation between urine and serum peptide specimens in studies comparing patients with endometriosis to controls. The initial research into urinary biomarkers as diagnostic tools for endometriosis is promising; however validation of these studies in larger sample sizes, of various populations, is required prior to their applicability into the clinical setting.

Peritoneal Fluid Biomarkers

Peritoneal fluid contains a wide variety of cells including macrophages, lymphocytes, and mesothelial cells [48]. These cells, particularly macrophages, are known to secrete high levels of cytokines. Peritoneal fluid cytokines may have an important role not only in the pathogenesis of endometriosis but also as a reflection of disease presence. This makes peritoneal fluid a useful specimen for potential diagnosis, identification of targets for therapy, and monitoring response to treatment. Unfortunately, collection of peritoneal fluid must be done at the time of surgery, making it less appealing than alternative sources of biomarkers including serum, saliva, and urine. While some have theorized an ultrasound-guided approach to aspiration of peritoneal fluid, this has never been completed clinically [49]. The recommended procedure as described by the WERF working group is to aspirate peritoneal fluid using a suction or syringe, following entry at the time of laparoscopy. It may be necessary to perform peritoneal lavage with 10 mL of sterile normal saline, if limited fluid is available [50]. Although many peritoneal biomarkers have been identified and studied in the literature, to date there has been no single biomarker or panel consistently identified and made clinically available for reliable diagnosis (Table 2).

Many biomarkers have been shown to have differing levels of expression in patients with endometriosis, but it remains difficult to determine what role these alterations play in the pathogenesis of endometriosis. With the complex interactions of cytokines and various other cells involved in the inflammatory response, alterations may contribute to the development of disease or reflect a change in the peritoneal environment caused by its progression.

Table 2 Endometriosis peritoneal fluid biomarkers

Marker	Assay specifics	Patient groups (*n*)	Detected value (*p* value)	Study
Tumor necrosis factor-α	Cut-off 20 pg/mL Sensitivity, 96% and specificity, 95% Interassay variation coefficient: <5% Intra-assay variation coefficient: <3% Lowest limit of sensitivity: 180 fg/mL	Control[A] (12) Endometriosis[A] (28) Control[A] (10) No endometriosis + infertility[A] (20) Endometriosis + fertility[A] (20) Endometriosis + infertility[A] (28) Superficial endometriosis[A] (7) Deep endometriosis[A] (6)	0 pg/mL ($p < 0.001$) 54.83 pg/mL ($p < 0.001$) 0 pg/mL ($p < 0.05$) 0 pg/mL ($p < 0.05$) 0 pg/mL ($p < 0.05$) 0 pg/mL ($p < 0.05$) 1.3 pg/mL 3.6 pg/mL	Bedaiwy et al. [51] Skrzypczak et al. [52] D'Hooghe et al. [53]
RANTES	Kit sensitivity: 2 pg/mL	Control[A] (55) Endometriosis stages I and II[A] (18) Endometriosis stages III and IV[A] (59)	11 pg/mL 22.5 pg/mL ($p > 0.1$) 35.5 pg/mL ($p < 0.001$)	Bersinger et al. [54]
Interleukin-6	Kit sensitivity: 0.7 pg/mL Interassay variation coefficient: <5% Intra-assay variation coefficient: <3% Kit sensitivity: 0.7 pg/mL	Control[A] (22) Endometriosis[A] (28) Control[A] (10) No endometriosis + infertility[A] (20) Endometriosis + fertility[A] (20) Endometriosis + infertility[A] (28) Superficial endometriosis[A] (7) Deep endometriosis[A] (6)	24.88 pg/mL (p = NS) 39.32 pg/mL (p = NS) 0 pg/mL ($p < 0.05$) 0 pg/mL ($p < 0.05$) 110 pg/mL ($p < 0.05$) 0 pg/mL ($p < 0.05$) 3.3 pg/mL 21.6 pg/mL	Bedaiwy et al. [51] Skrzypczak et al. [52] D'Hooghe et al. [53]
IFN-γ	Lowest limit of sensitivity: 3 pg/mL	Superficial endometriosis[A] (4) Deep endometriosis[A] (4)	0 pg/mL 6.7 pg/mL	D'Hooghe et al. [53]
Interleukin-1α	Kit sensitivity: 0.5 pg/mL	Control[B] (20) Early endometriosis: stages I and II[B] (38) Advanced endometriosis: stages III and IV[B] (26)	<0.5 pg/mL ($p < 0.0001$) 7.8 pg/mL ($p < 0.001$) 16.6 pg/mL ($p < 0.001$)	Kondera-Anasz et al. [55]

Interleukin-1β	Kit sensitivity: 1 pg/mL Interassay variation coefficient: <5% Intra-assay variation coefficient: <3%	Control[A] (25) Endometriosis[A] (35) Control[A] (10) No endometriosis + infertility[A] (20) Endometriosis + fertility[A] (20) Endometriosis + infertility[A] (28)	4.3 pg/mL (p = NS) 3.53 pg/mL (p = NS) 0.9 pg/mL 3.5 pg/mL 0.9 pg/mL 3.2 pg/mL	Bedaiwy et al. [51] Skrzypczak et al. [52]
Interleukin-1sRII	Kit sensitivity: 10 pg/mL	Control[B] (20) Early endometriosis: stages I and II[B] (38) Advanced endometriosis: stages III and IV[B] (26)	131.1 pg/mL ($p < 0.0001$) 57.3 pg/mL ($p < 0.0001$) 116.4 pg/mL ($p < 0.0001$)	Kondera-Anasz et al. [55]
Interleukin-1Ra	Kit sensitivity: 22 pg/mL	Control[B] (20) Early endometriosis: stages I and II[B] (38) Advanced endometriosis: stages III and IV[B] (26)	263.8 pg/mL ($p < 0.0001$) 181.6 pg/mL ($p < 0.0001$) 620.8 pg/mL ($p < 0.0001$)	Kondera-Anasz et al. [55]
Interleukin-8	Kit sensitivity: 10 pg/mL Interassay variation coefficient: <5% Intra-assay variation coefficient: <3% Kit sensitivity: 2 pg/mL	Control[A] (25) Endometriosis[A] (37) Control[A] (10) No endometriosis + infertility[A] (20) Endometriosis + fertility[A] (20) Endometriosis + infertility[A] (28) Control[A] (55) Endometriosis stages I and II[A] (18) Endometriosis stages III and IV[A] (59)	6.06 pg/mL ($p = 0.01$) 14.59 pg/mL ($p = 0.01$) 1 pg/mL ($p < 0.01$) 0 pg/mL ($p < 0.01$) 4 pg/mL ($p < 0.01$) 16 pg/mL ($p < 0.01$) 11.2 pg/mL 15.2 pg/mL ($p > 0.1$) 17.6 pg/mL ($p < 0.001$)	Bedaiwy et al. [51] Skrzypczak et al. [52] Bersinger et al. [54]
Interleukin-10	Lowest limit of sensitivity: 2 pg/mL	Superficial endometriosis[A] (5) Deep endometriosis[A] (6)	3.2 pg/mL 7.9 pg/mL	D'Hooghe et al. [53]
Interleukin-13	Kit sensitivity: 32 pg/mL	Control[A] (20) Endometriosis[A] (28)	0 pg/mL (p = NS) 1.2 pg/mL (p = NS)	Bedaiwy et al. [51]

(continued)

Table 2 (continued)

Marker	Assay specifics	Patient groups (*n*)	Detected value (*p* value)	Study
Interleukin-15	Kit sensitivity: 0.5 pg/mL	Control[B] (18) Endometriosis stage I[B] (20) Endometriosis stage II[B] (14) Endometriosis stage III[B] (14) Endometriosis stage IV[B] (7)	2.1 pg/mL ($p < 0.001$) 2.9 pg/mL 2.6 pg/mL 2.4 pg/mL ($p = 0.01$) 2.4 pg/mL ($p = 0.006$)	Arici et al. [56, 57]
Interleukin-16	NA	Control[A] (22) Endometriosis[A] (22)	778.1 pg/mL (p = NS) 539.4 pg/mL (p = NS)	Zhang et al. [58, 59]
Interleukin-18	Kit sensitivity: 8.0 pg/mL	Control[A] (22) Endometriosis[A] (22)	653.4 pg/mL ($p = 0.016$) 144.8 pg/mL ($p = 0.016$)	Zhang et al. [60]
TGF-β-1	Lowest limit of sensitivity: 5 pg/mL	Superficial endometriosis[A] (7) Deep endometriosis[A] (6)	0 pg/mL 0 pg/mL	D'Hooghe et al. [53]
Fas ligands	Intra-assay variation coefficient: 5.5% Interassay variation coefficient: 6.9%	Control[B] (29) Endometriosis stages I and II[B] (32) Endometriosis stages III and IV[B] (25)	81 pg/mL 80.5 pg/mL (p = NS) 166.2 pg/mL ($p < 0.05$)	Garcia-Velasco et al. [61]
Leptin	Assay minimum detection limit: 0.2 ng/mL	Control[A] (15) Endometriosis[A] (13)	17.5 ng/mL ($p = 0.005$) 35.9 ng/mL ($p = 0.005$)	Matarese et al. [62]

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A-median

B-mean

Gupta S, Agarwal A, Sekhon L, Krajcir N, Cocuzza M, Falcone T. Serum and peritoneal abnormalities in endometriosis: potential use as diagnostic markers. Minerva Ginecol. 2006 Dec;58(6):527–51

While Sampson's theory of retrograde menstruation persists as a plausible cause of endometriosis, it may only be a portion of a larger picture involving a disrupted immune response. Failure of the immune system to eliminate ectopic endometrial cells could help further explain the pathogenesis of disease. Significant changes have been observed in natural killer cells' characteristics and decreased activity [63]. Changes have also been reported in both B and T cell concentration and function, lending further support to the investigation of peritoneal fluid cytokines, and other immunological markers, and their roles in endometriosis [48].

Cytokines

Tumor Necrosis Factor-alpha

Despite the conflicting evidence available for peritoneal biomarkers in endometriosis, tumor necrosis factor alpha (TNF-α) is one of the more consistent cytokines identified in peritoneal fluid reported in literature. TNF-α is produced by a variety of cells, including neutrophils, lymphocytes, macrophages, and NK cells. Through the activation of helper T cells and various other cytokines, it functions to induce an inflammatory response [48]. TNF-α also has a proven role in angiogenesis, further supporting the proliferation and growth of endometriosis [63].

Multiple studies have shown elevated TNF-α level in samples of peritoneal fluid from women with endometriosis, compared to controls [48, 64]. Some studies report a diagnostic accuracy that rivals CA-125 [48]. One study reported a 99% area under the curve, indicating its discriminatory power [51].

RANTES

Regulated on activation, normal T-cell expressed and secreted (RANTES) is secreted from epithelial and mesenchymal cells and regulates inflammation in part by attracting natural killer cells, macrophages, and granulocytes [20, 48]. The peritoneal fluid level of RANTES has been reported higher in endometriosis patients, with some studies documenting a direct correlation with disease severity [65]. However, other studies have failed to show a difference [66].

Interferon- γ

Interferon gamma (IFN-γ) is known to activate macrophages, resulting in increased release of additional cytokines involved in the immune response [67]. It is also suggested that when present with interleukin-6 (IL-6), it may increase cellular adhesion molecules. Literature on the role of IFN-γ in endometriosis remains inconclusive. Some studies report no difference in the peritoneal IFN-γ of women with

endometriosis from those without disease [67]. However, some more recent data suggests there may be a role of IFN-γ as an important mediator in endometriosis [68]. Various studies describe a decreased IFN-γ level in endometriosis [69]. It is thought that this diminished expression could be a reflection of the decreased immune response in patients with disease.

Interleukin-6

Interleukin 6 (IL-6) may be the most commonly studied cytokine as a biomarker of endometriosis. Nonetheless, there is still no consensus regarding its level in peritoneal fluid of patients with endometriosis. Multiple studies report an increased level when compared to controls [49, 70]. However, others observe no significant change [51]. One possible explanation for this discrepancy may be related to the observed increase of IL-6 with increased severity of disease. The level of IL-6 in peritoneal fluid was significantly increased in patients with moderate to severe endometriosis [49, 71], while in women with mild disease, there may be no observed change in value. This could potentially limit the findings of studies that do not stratify patient population for severity of disease. This may also limit the clinical application of IL-6 as a diagnostic tool, if disease cannot be detected at an early stage. However, one study identified IL-6 as the only biomarker evaluated to have discriminatory power for diagnosis of endometriosis [49].

Interleukin-8

Interleukin-8 (IL-8) is involved in macrophage recruitment. It is also believed to play a critical role in angiogenesis and implantation of endometriotic tissue [63, 64]. Endometriotic tissue itself is able to produce IL-8, in addition to secretion from macrophages and mesothelial cells [64]. Studies evaluating IL-8 levels in peritoneal fluid for the diagnosis of endometriosis have been inconclusive. Many demonstrate an elevated concentration of IL-8 in the peritoneal fluid of women with endometriosis [51, 64, 72]. The relationship of IL-8 with severity of disease has also been investigated with discrepant results. A positive correlation with severity of disease is reported by some [64]. Others cite an inverse relationship, with more pronounced elevations seen in lower stages of endometriosis [73].

Interleukin-10

Type 2 T-helper (Th2) cells are the main producers of interleukin-10 (IL-10), which suppresses cell-mediated immunity. The alteration of cytokines and immune function observed in women with endometriosis includes favoring of Th2 cells and their resultant cytokines [48, 63]. As expected with this shift in the cellular peritoneal environment, data shows that IL-10 peritoneal fluid levels are higher in women with

endometriosis compared to controls [49]. In studies, concentrations were higher for all stages of disease, with no correlation observed between quantitative level and severity of endometriosis [49, 74]. These findings support the idea that a weakened immune response may contribute in allowing implantation and progression of endometriosis.

Interleukin-13

Similar to IL-10, IL-13 is a cytokine secreted by Th2 cells, which acts to suppress macrophage and lymphocyte activation. It has been proposed that low levels of IL-13 in endometriosis may allow increased activity of macrophages, due to decreased inhibition, contributing to the overall inflammatory process of endometriosis [75]. Consistent with this theory, studies have demonstrated reduced amounts of IL-13 in peritoneal fluid of women with endometriosis compared to controls [75, 76]. This decrease is observed independent of time of cycle [75].

Interleukin-16

Interleukin-16 (IL-16) is another pro-inflammatory cytokine evaluated more recently for a possible role in endometriosis development. Interleukin-16 can activate T cells, monocytes, and macrophages, stimulating production of other cytokines including IL-1b, IL-6, and TNF-α [77, 78]. Few studies have investigated the levels of IL-16 in peritoneal fluid, with contradictory findings. No change in IL-16 concentration was observed in one study [58, 59], while others have shown an increase, particularly in patients with advanced stage disease [78].

Interleukin-18

Interleukin-18 (IL-18) is produced predominately by activated macrophages. It has also been shown to be produced from ovarian tissue and endometrium. As a complex immunoregulatory cytokine, IL-18 is known to stimulate both Th1 and Th2 immune responses, inducing the release of interferon-γ, TNF-α, IL-4, IL-5, and IL-13 among others [56, 57, 60]. Given its role in various aspects of the immune response, IL-18 has become an obvious target for investigation in the development of endometriosis. Data on IL-18 levels in peritoneal fluid vary. Multiple studies have reported higher amounts of IL-18 in peritoneal fluid of women with endometriosis [56, 57, 79, 80]. However, some investigators have demonstrated lower peritoneal concentration of IL-18 in women with endometriosis, when compared to controls free of disease [60]. Still others observed no significant difference in levels between case and control groups [81, 82]. One possible cause of variation could be the anatomic site and type of endometrial lesion involved, as higher levels were noted in women with endometriosis-related peritoneal implants but not endometriomas [56, 57].

Other Biomarkers

TGF-ß-1

Transforming growth factor-β-1 (TGF-β-1) is thought to regulate cell proliferation and angiogenesis and inhibit natural killer cell activity [83]. This impedance of natural killer cell function may allow decreased clearance of endometrial cells in the peritoneal cavity from retrograde menstruation, allowing development of endometriosis [63]. In support of this theory, the concentration of TGF-β-1 in peritoneal fluid is significantly greater in women with endometriosis [64, 83]. Data shows these levels are further increased with increased stage of disease [64].

FAS Ligands

Fas ligand is a type II cell membrane protein involved in apoptosis [84]. Women with moderate to severe stage endometriosis have elevated concentrations of soluble Fas ligand, compared to controls. However, there has been no difference observed in early disease [61]. Higher levels of Fas ligand cause increased apoptosis, which may contribute to increased inflammation of the peritoneal cavity as seen in endometriosis. Increased apoptosis of immune cells could also prevent clearance of ectopic endometrial cells in the peritoneal cavity, allowing adhesion and proliferation of endometrial cells. In addition, Fas ligand stimulates IL-8 secretion, with the potential to further contribute to the development of endometriosis [61].

Leptin

Leptin is an adipocyte-derived protein. It has a role in the mediation of immune responses, inflammation, and angiogenesis, potentially linking it to the development of endometriosis. Peritoneal fluid levels of leptin are higher in women with endometriosis [62, 85, 86]. Levels may also have an inverse correlation with extent of endometriosis. Leptin concentrations in peritoneal fluid are higher in early-stage disease [62, 86]. However this relationship is inconsistent, with other studies showing a positive correlation to stage of disease [85]. These findings further support a possible role of leptin in the pathogenesis of endometriosis.

As discussed, there are multiple peritoneal fluid biomarkers identified that display altered concentrations in women with endometriosis. With no single cytokine or factor displaying a predictable and significant change, the most promising use of these peritoneal biomarkers may be in looking for a panel of markers. Utilized in combination, we may be able to find sufficient sensitivity to use peritoneal biomarkers as a noninvasive diagnostic tool. Since most of the studied markers are involved

in inflammatory and immune responses, it is likely that there would be confounding alterations with other disorders or comorbidities. Excluding patients with inflammatory disease may limit the population that could benefit from peritoneal fluid diagnosis on a routine basis. Further research into the roles of each marker in the pathogenesis of endometriosis and validation of previous studies are necessary before peritoneal fluid markers could be made clinically available.

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mRNA and miRNA Biomarkers for Endometriosis

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Abstract At present, the diagnosis of endometriosis is challenged by the absence of a clinically useful diagnostic biomarker(s). A biomarker is defined as a characteristic that can be objectively measured and evaluated to provide a unique and specific indicator of normal or pathological processes [1]. Alternatively, a diagnostic test, or disease classifier, does not necessarily contain unique disease characteristics, but nevertheless distinguishes those with disease and without, and can overlap with the disease biomarkers.

Keywords Endometriosis • Endometrium • Transcriptome • Epigenome • Biomarker • Classifier

Introduction

At present, the diagnosis of endometriosis is challenged by the absence of a clinically useful diagnostic biomarker(s). A biomarker is defined as a characteristic that can be objectively measured and evaluated to provide a unique and specific indicator of normal or pathological processes [1]. Alternatively, a diagnostic test, or disease classifier, does not necessarily contain unique disease characteristics, but nevertheless distinguishes those with disease and without, and can overlap with the disease biomarkers.

Endometriosis is a relatively widespread, benign, but debilitating gynecological disorder affecting reproductive age women. Its prevalence varies from 4 to 50% in

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different populations [2, 3], and it is reported in up to 50% of women with pelvic pain and/or infertility [4, 5]. It develops following the implantation of endometrial tissue in ectopic (outside of endometrium) locations, most frequently peritoneum, pelvic organs, and surfaces [4]. Severity of the clinical manifestation does not always correlate with disease burden at surgery [6], thus making the diagnosis of endometriosis based on clinical features challenging.

Currently, the gold standard to diagnosis and stage surface disease is direct visualization of lesions during surgery, confirmed by pathological evaluation of excised or biopsied lesions [4]. Intraoperatively, endometriosis is staged according to the revised American Fertility Society (AFS) classification system [7]. Time to definitive diagnosis can be delayed up to 7–11 years for several reasons, including an initial trial of empiric medical therapy, misdiagnosis, denial, and dismissal of pain as a normal event during menses, as well as high risks and costs associated with surgery [8–10]. Thus developing a noninvasive or minimally invasive/office-based biomarker of endometriosis and potentially disease stage would be advantageous to shorten the time to diagnosis, initiate timely therapies, and detect disease recurrence at the earliest stage. An ideal marker would be measured reliably with high sensitivity and specificity in serum or other body fluids or obtained by endometrial biopsy and is menstrual cycle independent in women with versus without endometriosis.

Currently, endometriosis biomarker discovery includes blood, urine, peritoneal fluid, and endometrial tissue-based approaches. Noninvasive diagnostic methodology such as MRI and pelvic ultrasound are not ideal to diagnose peritoneal endometriosis due to their low sensitivity and specificity [11]. While several studies have evaluated a variety of serum and urine biomarkers, none can be reliably utilized clinically to date (see relevant chapters herein; [12]). Though diagnosing endometriosis by blood or urine biomarkers is highly desirable, the specificity of such biomarkers may be compromised by other disorders with similar pathophysiologic features such as estrogen dependence or inflammation. An alternative approach is to evaluate eutopic endometrium, obtained via endometrial aspiration/biopsy in the office setting.

A Systems Biology Approach to Endometriosis Biomarker Discovery

In the era of systems biology, whole-genome microarray analysis has been a major approach used for biomarker discovery, with RNA sequencing (RNAseq) technology becoming more widely utilized [13, 14]. The RNAseq approach to transcriptome profiling is a relatively new method that uses deep-sequencing technologies [13]. It offers certain advantages over microarray technology despite its higher cost and more sophisticated data storage and analysis [14]. RNAseq and microarray analyses performed on the same RNA samples demonstrated high correlation in gene expression profiles generated by both methods; however the RNAseq technology showed superiority in terms of detection of low abundance but biologically important transcripts, more differentially expressed genes with high fold change, differentiating biologically critical isoforms, and allowing the identification of

genetic variants [14]. These large-scale molecular/genomic approaches allow identification of genes and pathways not previously identified in tissues and isolated cells and the development of disease-specific biomarker(s) and offer the promise of identifying unique targets leading to new and individualized therapies, as advances in ovarian cancer treatment, e.g., have recently demonstrated [15, 16]. Compared to microarray expression profiles that were used to classify disease states almost a decade ago [17], RNAseq technology has yet to be fully integrated into human endometrial research, as the vast majority of studies have been focused on the embryo receptivity transcriptome in cattle and other domesticated species [18, 19]. The first study on human endometrium that analyzed gene expression profiles of the pre-receptive versus receptive phases of the natural cycle using RNAseq identified novel, previously unreported candidate genes [20]. To date, no studies on endometriosis utilizing this technology have been reported.

In the field of endometrial research, analysis of the endometrial transcriptome has been by far the most commonly applied "omics" approach beginning with the first publication in 2002 (reviewed in [21]). Endometrial whole tissue transcriptomic analysis has been performed in control subjects as well as in those with uterine disease states such as implantation failure, endometriosis, uterine fibroids, and endometrial cancer (reviewed in [21–23]. With regard to epigenomic analysis of endometrium, the most commonly used "omics" platform to date has been microRNA (miRNA) arrays [21].

From a historic perspective, early endometrial researchers analyzed normal human endometrium throughout the menstrual cycle and specifically during the window of implantation, followed by evaluation in the setting of various pathological conditions. Shortly thereafter, five groups published endometrial transcriptome expression in mid-secretory endometrium in healthy fertile women [24–28], followed by analysis of the endometrial transcriptome throughout the menstrual cycle [29, 30] reviewed in [22, 31]. However, despite using the same array platform, there were only nine genes that were significantly and similarly regulated during the window of implantation in at least four of the five reports (reviewed in [31]. These large differences between studies can be explained by differences in study design, subject characteristics, procedures for tissue collection, handling and processing, microarray platforms, hybridization conditions, and algorithms used for data analysis. These discrepancies potentially confound the identification of informative molecular markers. Consequently, the development of standard operating procedures (SOP) in the handling of human endometrial tissue used in biomarker discovery is a top priority [10].

Standard Operating Procedures

In order to provide high-quality biospecimens with well-characterized data, one must insure that "best practices" are followed [32]. Adherence to SOPs and "best practices" significantly reduces variations in protocols, with the goal of technical reproducibility and comparability of results, and facilitation of quality collaborative

research [33]. The University of California, San Francisco, NIH Human Endometrial Tissue and DNA Bank (http://obgyn.ucsf.edu/crs/tissue_bank/) was established in 1999 as a national resource specifically to foster collaborative research and has been collecting endometrium from healthy women and from women with uterine pathology, using well-described standard operating procedures (SOPs) [34]. This SOP specifically addressed tissue collection, processing, and long-term storage, which are crucial steps to ensure sample quality and minimization of variance due to non-biologic contributing factors (i.e., warm or cold ischemia, desiccation, etc.). Recently, the World Endometriosis Research Foundation reported the results of a global effort, the Endometriosis Phenome and Biobanking Harmonisation Project (EPHect), which developed consensus standards for tissue collection, processing, and storage in endometriosis research [33]. EPHect also developed consensus on standardization and harmonization of phenotypic surgical, clinical/covariate data and fluid biospecimen biologic sample collection methods in endometriosis research [35–37]. Adherence to these recommendations is essential for cross-study comparisons and reproducibility of research targets, including biomarker development.

Endometrial mRNA as Biomarkers for Endometriosis

Steroidogenesis in Endometrial Tissue

It is currently well established that normal endometrium differs from both ectopic and eutopic endometrium. Endometriosis is a steroid hormone-responsive disease that also possesses its own steroidogenic machinery [38]. Ectopic endometrium expresses high level of aromatase which allows for higher estrogen production and progression of ectopic lesions by paracrine mechanisms [39–41]. Additionally, human endometrium expresses transcripts for important enzymes of the steroidogenic pathway involved in the synthesis and metabolism of estrogens and progesterone [38, 42]. Dysregulation of some enzymes (decreased 3-beta hydroxysteroid dehydrogenase type I (HSD3B1) and HSD17B2 and increased HSD17B1 and aromatase) favors local production of estradiol in eutopic endometrium of women with endometriosis [38]; however, this was not shown in another study [42]. Elevated local estradiol levels may blunt progesterone actions within the endometrium, leading to the observed molecular phenotype of progesterone resistance [43].

Progesterone Resistance

Studies on endometrial stromal fibroblasts (eSF) from women with endometriosis showed a blunted response to decidualization stimuli [44, 45] and resistance to progesterone, due to lower levels of PGRA, PGRB [46, 47], and PGR coregulator

expression [48, 49], as well as dysregulation of specific pathways involved in eSF decidualization (e.g., PKA pathway, epidermal growth factor (EGF) pathway, cell cycle pathway, vascular endothelial growth factor (VEGF) pathway, G protein-coupled receptor signaling, Wnt pathway, and others) [43–45, 50]. In a time-course set of experiments, delayed estrogen-stimulated upregulation of PGR was observed, supporting the hypothesis that endometrial stromal cells from women with endometriosis are resistant to progesterone action [51]. At the whole tissue level, studies by Kao et al. [52] and Burney et al. [53] demonstrated dysregulation and decreased expression of progesterone-responsive genes in mid-secretory eutopic endometrium [52] and in the proliferative-to-early secretory (PE-to-ESE) endometrial transition [53]. As shown in normal endometrium, there is a downregulation of cell cycle-related genes highlighting the proliferative-to-secretory transition [30]. In eutopic endometrium from women with moderate-severe endometriosis, significant dysregulation of this transition was observed (consistent among all ESE samples from subjects with endometriosis), suggesting a phenotype of resistance to progesterone action in the setting of endometriosis [53]. Further analysis of progesterone target genes confirmed their dysregulation. Additionally, principal component analysis of specimens from women with endometriosis demonstrated clustering of ESE and PE samples [53].

Apoptosis, Proliferation, and Cell Survival

At the whole tissue level, several apoptosis and proliferation markers have been demonstrated in endometrium from women with endometriosis. In a comprehensive microarray analysis of endometrial tissue from women with versus without endometriosis throughout the menstrual cycle, Burney et al. [53] showed a delayed transition from the proliferative to secretory phase, represented by a proliferative phase genetic fingerprint including persistent expression of genes involved in mitosis and proliferation [53]. Eutopic endometrium from women with, but not without, endometriosis showed decreased expression of genes associated with inactivation of mitogen-activated protein kinase (MAPK) signaling cascades, such as ERBB receptor feedback inhibitor 1 (ERRFI1) (mitogen-inducible gene (MIG)-6/receptor-associated late transducer (RALT)/Gene33), which is a negative regulator of MAPK signaling, and regulator of G protein signaling 1 (RGS1), which is an activator of guanosine triphosphate(GTP)ases that rapidly switches off G protein-coupled receptor signaling pathways [53]. The anti-apoptotic gene, BCL-2, was upregulated in ESE from women with endometriosis, in agreement with other studies [54, 55], suggesting enhanced cell survival in the pathogenesis of endometriosis. Johnson et al. [56] revealed increased proliferation and decreased apoptosis in eutopic endometrium of women with versus without endometriosis [56]. These results support the possibility that an intrinsic abnormality in the eutopic endometrial cells from women with endometriosis predisposes the cells to survive, attach, invade, and establish a blood supply in the peritoneum or other areas.

Immunological and Inflammatory Factors

Endometriosis is known to be associated with an inflammatory peritoneal environment [57]. Several immune and inflammatory molecules have been examined in the setting of endometriosis and even assessed as possible biomarkers and therapy targets [11, 58]. Soluble markers measured in serum are discussed elsewhere in this publication. Endometrial expression of interleukin-8 (IL-8), which is responsible for chemotaxis of neutrophils and is a potent angiogenic agent, and its receptor are significantly higher in women with endometriosis and are involved in endometrial cell proliferation and attachment [57]. In a systematic review of different chemokines as marker of endometriosis, IL-8 appeared to be the most promising [59].

The Implantation Window

Both animal models of endometriosis and studies on human endometrium suggest progesterone resistance in eutopic endometrium and impaired endometrial receptivity. Ectopic placement of normal mouse endometrium resulted in decreased expression of progesterone-responsive and endometrial receptivity-associated genes, such as Hoxa10, Hoxa11, IGFBP1, and Kruppel-like factor 9 [60], as well as an increased PRAB and PRB-to-PRAB ratio. In baboons, induced endometriosis resulted in decreased eutopic endometrial expression of progesterone-regulated genes, including glycodelin and HOXA10 [50, 61].

Significant dysregulation of several receptivity markers in human endometrium was observed, including downregulation of αvβ3 integrin that normally signifies the implantation window [62, 63], the epithelial marker leukemia inhibitory factor (LIF) [64], HoxA10, HoxA11 [65], and IL-11 [64].

Kao et al. [52] used a two-way overlapping layer analysis to compare endometrial gene expression during the window of implantation (WOI, also MSE) in women with versus without endometriosis, which identified three unique groups of target genes. Group 1 target genes were upregulated in endometrium during the WOI in controls, but significantly decreased in endometriosis: IL-15, proline-rich protein, B61, Dickkopf-1 (DKK1), glycodelin, *N*-acetylglucosamine-6-*O*-sulfotransferase, G0S2 protein, and purine nucleoside phosphorylase. Group 2 genes were normally downregulated during the WOI, but significantly increased with endometriosis: semaphorin E, neuronal olfactomedin-related endoplasmic reticulum localized protein mRNA, and Sam68-like phosphotyrosine protein alpha. Group 3 had only a single gene, neuronal pentraxin II, normally downregulated during the window of implantation, which further decreased in endometrium from women with endometriosis. Burney et al. [53] also found dysregulation of several WOI-related genes in MSE from women with endometriosis, including CYP26A1, glutathione peroxidase (GPX)-3, decidual protein induced by progesterone (DEPP), Dickkopf (DKK)-1, and stanniocalcin1 [53].

The profound transcriptomic changes during the WOI in eutopic endometrium from women with endometriosis appear to create an inhospitable, if not hostile, environment for an implanting embryo, due to dysregulation of genes involved in embryonic attachment, stromal decidualization, immune function, and apoptotic responses, contributing together with angiogenic factors, dysregulated progesterone receptor, and aromatase, to the pathophysiology of endometriosis-associated infertility [52, 53, 66].

Neuroangiogenesis

In order to survive in the peritoneal environment, endometriotic lesions must establish a de novo blood supply. Neoangiogenesis occurs concomitantly with neuronal sprouting, collectively known as neuroangiogenesis [67] and has been postulated to be supported by high levels of VEGF and other angiogenic factors detected in peritoneal fluid from women with endometriosis [68]. Compared with expression in healthy endometrium, endometrium from women with endometriosis expresses higher levels of VEGF [69]. A transcript for pleiotrophin, an angiogenesis associated peptide, was significantly upregulated in eutopic endometrium from women with severe endometriosis [70].

Detection of neuronal fibers in eutopic and ectopic endometrium is relatively novel and has been proposed as a biomarker for endometriosis. Described initially in human endometriotic lesions and thought to be responsible for dysmenorrhea [71], nerve fibers were detected in a mouse model of endometriotic lesion innervation [72]. At the level of protein expression, nerve fibers were repeatedly reported in endometrium from women with endometriosis, but not in those with endometritis, leiomyomata, or endometrial polyps [73–75]. At the level of gene expression, the axon signaling pathway is one of the pathways detected and dysregulated in eutopic endometrium from women with endometriosis in microarray studies [76]. Using laser capture microdissection of endometrial cell types, Matsuzaki et al. [77] identified upregulation of the following genes putatively involved in the endometriosis-related pain in subjects with endometriosis: tyrosine kinase receptor B (TRkB) in epithelial cells and serotonin transporter (5HTT) and mu opioid receptor (MOR) in stromal fibroblasts [77].

Endometrial Transcriptome Dysregulation in Endometriosis

Table 1 presents a list of microarray studies performed using human endometrial tissue obtained from women with endometriosis. Notably, these studies used samples from different menstrual cycle phases, location of endometrial tissue (eutopic versus ectopic), and used different microarray platforms.

Table 1 Gene expression microarray studies performed on human endometrial tissue from women with endometriosis (modified from Aghajanova et al. 2010)

Study	Cycle phase	Type of endometrial tissue	Endometriosis stage/comments	Array Reference
Kao et al. (2003)	MSE	Eutopic	Mild/moderate	Affymetrix Genechip Hu95A
Matsuzaki et al. (2004)	PE, SE	Eutopic vs. ectopic (deep endometriosis)	I–IV	Clontech Atlashuman 1.2 cDNA expression array
Absenger et al. (2004)	PE, SE	Eutopic, ectopic		Affymetrix Genechip Hu95A
Matsuzaki et al. (2005)	LPE, ESE, MSE, LSE	Eutopic (deep endometriosis)	Not defined/ epithelial vs. stromal cells	Clontech Atlashuman 1.2 cDNA expression array
Wu et al. (2006)	PE, SE	Eutopic vs. ectopic	II–IV	House-made
Mettler et al. (2007)	PE	Eutopic unmatched vs. ectopic		Clontech Atlashuman 1.2 cDNA expression array
Eyster et al. (2007)	PE	Eutopic vs. ectopic	I–IV	CodeLink Whole Human Genome Bioarrays
Burney et al. (2007)	PE, ESE, MSE	Eutopic	Moderate/severe	Affymetrix Human U133-Plus 2.0
Sherwin et al. (2008)	LSE	Eutopic	Minimal/mild, moderate/severe	Custom-made array, University of Cambridge
Aghajanova, Giudice (2011)	PE, ESE, MSE	Eutopic	Mild, severe	Affymetrix Human U133-Plus 2.0
Fassbender et al. (2012)	Menstrual, ESE	Eutopic	Minimal/mild, moderate/severe	Affymetrix GeneChip Human Gene 1.0 ST
Tamaresis et al. (2014)	PE, ESE, MSE	Eutopic	Minimal/mild, moderate/severe	Affymetrix Human U133-Plus 2.0

PE proliferative endometrium, *ESE* early secretory endometrium, *MSE* mid-secretory endometrium

Cycle Dependent

The first microarray analysis of human eutopic endometrium from women with versus without endometriosis was performed by Kao et al. in the mid-secretory phase [52]. The authors found 91 significantly upregulated and 115 significantly downregulated genes (>2-fold). Dysregulation of several progesterone-regulated genes, such as interleukin-15, proline-rich protein, B61, Dickkopf-1, glycodelin, *N*-acetylglucosamine-6-*O*-sulfotransferase, and G0S2, was reported and suggested dysregulation during the implantation window perhaps due to a compromised response to progesterone [52].

Subsequently, Burney et al. [53] investigated the endometrial transcriptome in women with versus without endometriosis throughout the menstrual cycle [53]. They demonstrated for the first time the molecular dysregulation of the proliferative (estrogen dominant)-to-secretory (progesterone and protein kinase A (PKA) dominant) transition in the endometrium of women with disease and an attenuated progesterone response suggestive of progesterone resistance in ESE transcriptome [53].

In an attempt to evaluate if mild and severe stages of peritoneal endometriosis may be molecularly distinct disorders as suggested by clinical differences [78–80], Aghajanova and Giudice [76] performed comparative microarray analysis on the endometrium from 19 mild and 44 severe endometriosis subjects throughout the cycle (PE, ESE, MSE) [76]. The analysis revealed dysregulation of progesterone and/or cyclic adenosine monophosphate (cAMP)-regulated genes (downregulation of IHH, SST, and TAGLN in ESE and upregulation of DKK1, MAO, IL15, and IL1R1 in MSE in severe versus mild endometriosis) and genes related to thyroid hormone action and metabolism (upregulation of DIO2 and downregulation of TRH in severe versus mild endometriosis). Some of the differences between severe and mild endometriosis were related to the EGFR signaling pathway, with the greatest upregulation of EGFR in severe versus mild disease in ESE. The extracellular matrix proteoglycan versican, responsible for cell proliferation and apoptosis, was the most highly expressed gene in severe versus mild disease.

Fassbender et al. [81] used a slightly different approach, simultaneously investigating the differential gene expression and protein expression in menstrual and early luteal endometria in women with and without endometriosis [81]. Their choice of menstrual endometrium was rationalized by studying the endometrium that is shed and transported to the peritoneal cavity during retrograde menstruation resulting in the establishment of lesions. This study revealed no differentially expressed genes in menstrual endometrium from women with endometriosis compared to controls. One explanation for the lack of differentially expressed genes may be heterogeneity of the control group that included women with fibroids, benign ovarian cysts, and hydrosalpinges. Of note, it was shown previously by Sherwin et al. [82] that the late secretory endometrial transcriptome in women with versus without endometriosis is uninformative and not useful in the development of a diagnostic test for endometriosis [82].

Cycle Independent

A comprehensive microarray study on eutopic endometrial samples from the proliferative and secretory cycles phases (cycle phase allocation based on serum progesterone levels) suggested that Cyr61 (cysteine-rich, angiogenic inducer, 61; CCN1) is a cycle-independent biomarker for endometriosis. This was based on its consistent upregulation in 18 of 20 samples analyzed at the gene and protein levels and confirmed in a nude mouse xenograft model of endometriosis [42]. It was also upregulated in endometriotic lesions, and its role in endometriosis pathogenesis was speculated to be associated with facilitating adhesion and angiogenesis [42]. Also, increased levels of CYR61 mRNA were found in the endometrium of baboons with induced endometriosis, as well as in blood vessels in ectopic and eutopic endometria correlated with VEGF levels [83]. Interestingly, this increase of CYR61 mRNA in eutopic endometrium of baboons was detected following peritoneal inoculation with menstrual endometrium, suggesting a feedback mechanism from induced ectopic lesions to gene expression patterns in the eutopic endometrium [83].

Molecular Classifiers to Diagnose and Stage Endometriosis

For the reasons described above in this and other chapters, there is an urgent need for a minimally invasive diagnostic test for endometriosis. An endometrial diagnostic assay is preferably obtained in the proliferative phase, to avoid concerns regarding interruption of an unanticipated pregnancy. Another preferred characteristic of a classifier would be its menstrual cycle independence and ability to detect disease at all stages (minimal to severe). Importantly, a classifier should delineate endometriosis from other pelvic pathology, such as uterine fibroids, adenomyosis, hydrosalpinx, endometritis, and endometrial polyps, which often accompany endometriosis and have been shown to significantly affect the eutopic endometrial gene expression signature [84–86]. Therefore, the specimens involved in the process of biomarker discovery and validation should be carefully screened for coexisting pathology, and unequivocal endometriosis samples should be ideally used.

May et al. [87] performed a systematic review of over 200 potential endometrial biomarkers, including hormones and their receptors (n = 29), cytokines (n = 25), factors identified through proteomics (n = 8), and histology (n = 10), with reported sensitivity and specificity in only 32 articles ranging from 0 to 100% [87].

Recently, Giudice and colleagues reported the development of menstrual cycle phase-specific classifiers with high accuracy in the detection of both endometriosis and staging of disease [88]. They used whole-genome microarray data involving 148 endometrial specimens from women with confirmed endometriosis or other benign surgically confirmed gynecologic pathology (i.e., leiomyomata, endometrial polyp, hydrosalpinx) and women with no uterine pathology (confirmed surgically) throughout the menstrual cycle, all collected and processed using well-established SOPs.

The performance of the classifier was evaluated on an independent sample set. Classification of samples from women with and without endometriosis involved two binary decisions based on expression of specific genes. The first decision was to distinguish the presence or absence of uterine/pelvic pathology; the second decision was differentiation of endometriosis from no endometriosis, followed by classification of endometriosis according to disease severity (minimal/mild or moderate/severe). Moreover, the study reported (1) menstrual cycle phase unrestricted classifiers diagnosing samples in all cycle phase categories, (2) phase-restricted classifiers diagnosing samples in both PE and ESE, and (3) phase-specific classifiers (PE, ESE, or MSE) diagnosing samples in the corresponding cycle phase. Best performing classifiers identified endometriosis with 90–100% accuracy, were cycle phase specific or independent, and used relatively few genes to determine disease and severity [88]. Interestingly, a relatively small number of genes (less than 100) were sufficient to separate endometriosis from other uterine pathologies and to classify disease by severity. In particular, PE and ESE phase-specific disease classifiers achieved 100% accuracy using less than 100 genes for each disease classification decision. In addition, gene expression and pathway analyses revealed immune activation, altered steroid and thyroid hormone signaling/metabolism, and growth factor signaling in the endometrium of women with endometriosis, confirming earlier findings described above. To date, this study [88] represents the first and only study involving the classifier analysis of genomic data from healthy and diseased endometrium for the detection and staging of pelvic endometriosis with high accuracy. Validation of the classifier in a large multisite independent cohort is necessary.

Endometrial miRNA as Biomarkers for Endometriosis

MicroRNAs (miRNAs) are highly conserved endogenous short 19–25-nucleotide-long noncoding RNAs, which, in general, repress transcription of the target mRNA [89, 90]. miRNAs function as posttranscriptional regulators of gene expression and operate through RNA interference, either degrading or translationally repressing target mRNAs [91, 92]. A single miRNA can regulate multiple, up to 1000 genes, and several miRNAs can target the same gene.

Endometrial miRNAs Dysregulated in Endometriosis

A recent review by Gilabert-Estelles [93] summarized the available literature on miRNAs in gynecological pathology, including three gynecological malignancies (endometrial, cervical, and ovarian cancer), and endometriosis, which shares some key molecular features with cancer. This review suggested not only an important role for miRNAs in the pathogenesis of endometriosis but also the possibility of miRNA as a disease biomarker [93].

Table 2 Global miRNA profiling studies performed on human endometrial tissue from women with endometriosis

Study	Cycle phase	Type of endometrial tissue	Endometriosis stage/comments	Array Reference
Pan et al. (2007)	ESE, MSE	Ectopic, eutopic	Stage III	mirVana miRNA Array
Burney et al. (2009)	ESE	Eutopic	Severe	miRCURYTM LNA Array (version 10.0, Exiqon)
Ohlsson Teague et al. (2010)	PE, SE	Ectopic, eutopic	Stages II–IV	mirVana miRNA Array
Filigheddu et al. (2010)	PE	Ectopic (endometrioma), eutopic	Unknown	mirVana miRNA Array
Hawkins et al. (2011)	PE	Ectopic (endometrioma), eutopic	Unknown	Illumina's Human WG-6 version 2.0 BeadChips (Illumina)
Laudanski et al. (2013)	PE	Eutopic	Severe	TaqMan MicroRNA Array Cards
Shi et al. (2014)	PE	Ectopic, eutopic	Unknown	miRCURY LNA™ microRNA Array (v. 14.0; Exiqon)
Saare et al. (2014)	PE, SE	Ectopic, eutopic, matched healthy surrounding tissue	Stages III–IV	High-throughput miRNA sequencing

PE proliferative endometrium, *ESE* early secretory endometrium, *MSE* mid-secretory endometrium, *SE* secretory endometrium

MicroRNA regulation and dysregulation in eutopic endometrial tissue from women with endometriosis has been investigated within the past decade. Pursuant to their study on the global endometrial transcriptome that revealed incomplete transitioning from PE to ESE in the setting of moderate to severe endometriosis [53], Burney and colleagues performed global miRNA array analysis of the same tissue samples, allowing parallel miRNA–mRNA expression profiling [94]. Members of the miR-9 and miR-34 families were downregulated in ESE from women with endometriosis (Table 2). Cross-referencing of the predicted mRNA targets with the differentially expressed genes in ESE from women with versus without endometriosis revealed 156 genes, which upon pathway analysis were associated with the biological processes of cell death, cell cycle, and cellular assembly and organization [94]. Earlier, Pan et al. [95] performed paired analysis of eutopic and ectopic endometrium and found differential expression of miRNAs in these tissues [95].

It is notable that miRNAs in human endometrial tissue exhibit cycle phase variation and endometrial cells in culture are regulated by ovarian steroids, thus likely explaining variations in findings among studies [95, 96]. A comprehensive miRNA microarray study identified 22 dysregulated miRNAs in paired ectopic and eutopic

endometrial tissue samples (14 upregulated, miR-145, miR-143, miR-99a, miR-99b, miR-126, miR-100, miR-125b, miR-150, miR-125a, miR-223, miR-194, miR-365, miR-29c, and miR-1, and 8 downregulated, miR-200a, miR-141, miR-200b, miR-142-3p, miR-424, miR-34c, miR-20a, and miR-196b miRNAs), with corresponding mRNA expression mapping which showed that 673 of the putative targets were also differentially expressed in ectopic versus eutopic endometrium [97]. Furthermore, miR-483-5p and miR-629* were found to be downregulated in eutopic endometrium from women with endometriomas [98]. Microarray analysis demonstrated significant downregulation of miR-200a, miR-200b, miR-200c, and miR-182 in ectopic endometrium, while miR-202 expression was increased in ectopic versus eutopic endometrium [99]. A study involving miRNA sequencing showed upregulation of miR-34c, miR-449a, miR-200a, miR-200b, and miR-141 in peritoneal endometriotic lesions compared to healthy peritoneal tissues [100]. They also showed increased expression of miR-200 family target gene E-cadherin in endometriotic lesions compared to healthy tissues [100]. Inhibition of apoptosis in endometriotic cells resulted in downregulation of miR-183 and miR-196b [101, 102]. Upregulation of microRNA 21 (MIR21) and DICER1 transcripts was observed in a comparative study of endometrial transcriptomes of severe versus mild endometriosis, again suggesting a role for microRNAs in the pathogenesis of severe versus mild disease, potentially through regulation of gene silencing and epigenetic mechanisms [76].

To date, only two studies have applied next-generation sequencing/deep sequencing (RNA sequencing, in which all miRNAs, including unknowns, are sequenced) for identifying miRNA expression profiles in human endometrium from women with endometriosis [103, 104]. miRNA profiling between endometriomas and eutopic endometrium from women without endometriosis, combined with transcriptome profiling and in silico microRNA targeting predictions, revealed multiple biologically important pathways dysregulated in endometriomas potentially through functional microRNA [104]. miR-29c, which targets several extracellular matrix genes, was validated in endometriomas [104].

Based on the above data, identification of dysregulated endometrial miRNAs was recently suggested for the development of potential biomarkers identifying endometriosis [105]. Further studies however are warranted to identify a panel of miRNAs that are potentially diagnostic for the disease.

Circulating miRNAs in Endometriosis

First described as a noninvasive biomarker for diffuse large B cell lymphoma [106], circulating miRNAs have since gained increasing interest. Alterations in circulating microRNA profiles have been linked to various disease states and tumors [93, 107–110], including ectopic pregnancy [111]. Correlations between serum and cancer tissue microRNA profiles [112, 113], suggest that microRNAs may be released from tissues and shed into the circulation [114]. Jia et al. [114] performed microarray-based microRNA expression analysis of plasma samples from women

with and without histologically-confirmed stage III–IV endometriosis in a Chinese population [114]. They found and validated significantly reduced plasma levels of miR-17-5p, miR-20a, and miR-22, suggesting the promise of plasma miRNA evaluation as a noninvasive diagnostic of endometriosis. In another study, serum miR-199a and miR-122 were elevated and miR-145*, miR-141*, miR-542-3p, and miR-9* were decreased in serum from women with endometriosis patients compared to controls [115]. Also, miR-16, miR-191, and miR-195 levels evidence elevation in women with versus without disease [116]. Collectively, these data provide a strong foundation for future studies aimed at the identification of dysregulated microRNA(s) for the nonsurgical diagnosis of endometriosis.

Conclusions

Endometriosis is a benign chronic and debilitating disease, which even now requires surgery for definitive diagnosis. Identification of mRNA or miRNA signatures of the disease provides bases for the development of biomarker(s). Technological advances have allowed for significant progress in the development of potential noninvasive diagnostics for endometriosis, laying the foundation for future validation in the clinical setting and furthermore in clinical trials.

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Proteomic Biomarkers for Endometriosis

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Abstract Endometriosis is a benign, estrogen-dependent gynecology disorder associated with pelvic pain and infertility. It is characterized by the presence of endometrial-like tissue outside the uterine cavity, mainly on the pelvic peritoneum and ovaries and in the rectovaginal septum and more rarely in the pericardium, the pleura, and even the brain. The etiology and pathogenesis remains unclear. The most accepted theory is Sampson's theory: retrograde menstruation. The gold standard of diagnosing endometriosis is through laparoscopy.

Proteomics research has found differentially expressed protein/peptides; however, till today we have not found a non- or semi-invasive test for endometriosis. To date, two most commonly applied technologies used in endometriosis research are surfaced-enhanced laser desorption ionization (SELDI)-time-of-flight (TOF) mass spectrometry (MS) and two-dimensional difference gel electrophoresis (2D DIGE). In this chapter we will discuss the proteomics technologies available and their advantages and disadvantages and critically describe the biomarker proteomics results in endometriosis.

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Abbreviations

2D DIGE	Two-dimensional difference gel electrophoresis
FTMS	Fourier transform mass spectrometry
HPLC	High-performance liquid chromatography
ICAT	Isotope-coded affinity tags
ICPL	Isotope-coded protein labeling
LC	Liquid chromatography
MALDI	Matrix-assisted laser desorption ionization
MS	Mass spectrometry
MudPIT	Multidimensional Protein Identification Technology
SCX	Strong cation exchanger
SELDI	Surface-enhanced laser desorption ionization
SILAC	Stable isotope labeling by amino acids in cell culture
TOF	Time of flight

Introduction

The importance of having a reliable biomarker, or a set of reliable biomarkers, for the diagnosis of specific diseases should be evident, especially when it can detect early stages of the disease. Additionally, biomarkers could also be applied to predict the outcome of a disease or to monitor and guide the therapy. Regarding the chemical composition of the biomarker(s), all kinds of biomolecules could serve as a valuable marker: this can be proteins, peptides, lipids, metabolites, and nucleic acids. As the various '-omics' strategies, including proteomics, peptidomics, lipidomics, and metabolomics, are essentially based on various mass spectrometry methods, mass spectrometry (MS) has become the key analytical tool for biomarker discovery.

An important issue in the biomarker discovery research is that the most relevant modifications are found at the site of the affected tissue itself where these molecules are obviously present at their highest concentration. As a result, the analysis of biopsies is still the best starting point to find relevant molecular modifications. However, the ultimate goal behind biomarker discovery research is to identify relevant biomolecules in easily obtained patient samples (particularly blood, urine, feces). This automatically implies that these molecules should be released from the affected site and should be transportable (water-soluble or attached to a transport

protein). The amount released should be appropriate to deal with the substantial dilution (e.g., dilution in 5 L blood) in the final sample. There is an increasing evidence that a panel of biomarkers, instead of a single biomarker, dramatically improves the quality in terms of selectivity and specificity. This requires advanced data processing for accurate weighing of each potential biomarker (see further). Obviously, all these considerations are not only valuable in the field of endometriosis but apply to the whole field of biomarker discovery. In following chapters, we will focus on common proteomics strategies and studies described in endometriosis.

Proteomics Strategies for Biomarker Discovery

Various strategies have been applied and are applied to screen for differences in the protein content related to a specific disease or a specific disease state. Gel electrophoresis is a common technique to analyze the protein content of biological samples, but has only a very limited resolving power and therefore insufficient to analyze complex protein mixtures. This does not apply to 2D gel electrophoresis where two dimensions are used (separation based on the isoelectric point combined with separation based on the molecular weight). 2D gel electrophoresis can separate up to several 1000s of protein spots and is probably one of the oldest methods applied in high-throughput proteomics. The multiplexed variant (2D DIGE), in which multiple samples (up to three) are combined, allows parallel analysis of different samples and has been proven to be very effective and very popular. Although this approach has several drawbacks, it has several strong merits including simplified data analysis, and it is still used (see further). The idea to separate proteins using two dimensions has also been proposed in a chromatographic format using separation based on isoelectric point (isoelectric focusing LC) and on molecular weight (Beckman PF 2D), thereby copying exactly the strategy used in 2D gel electrophoresis.

Mass Spectrometry Is the Key Analytical Tool in Biomarker Discovery

In the strategies mentioned above, mass spectrometry only plays a role in the identification of the detected differences in protein content. However, in most currently applied strategies, mass spectrometry itself is used to detect these differences. This differential analysis is performed by comparison of the signal intensities of the measured ions in mass spectrometry and is used as a parameter to evaluate the abundance. These signals can come from intact proteins (such as used in SELDI-TOF MS; see further) or—more commonly—from labeled or unlabeled peptides generated from these proteins.

As sample complexity severely affects the outcome of the MS analysis, an impressive collection of methods have been proposed (e.g., multiple liquid chromatography (LC) steps) developed to reduce this sample complexity. Separation strategies using multiple dimensions improve the resolution, sensitivity, and overall quality of the obtained data, but induce less straightforward data processing. The first dimension could be anything from an affinity step (e.g., immunoprecipitation, affinity beads) to solid-phase extraction, physical separation (centrifugation, ultrafiltration, etc.), ion-exchange chromatography, reversed-phase chromatography at high pH, or even gel slices from a 1D gel electrophoresis. The final dimension typically separates the peptide mixture by reversed-phase chromatography at low pH. An example of an early 2D LC-MS-based proteomics strategy is the original multidimensional protein identification technology (MudPIT) method (see further), combining an initial digestion of the sample and a separation by ion-exchange and reversed-phase chromatography.

Labeled and Unlabeled Methods

When in proteomics the focus is set on biomarker discovery, it is required not only to identify but also to quantify proteins in different samples in order to obtain a more detailed picture of the differences between various conditions, e.g., healthy versus disease or mutant versus wild type. This quantitative proteomics can be obtained by comparing samples which have been labeled (labeled methods) with dedicated tags. Labeling can be performed at the protein level or at the peptide level (after digestion of the protein). Each method has its own benefits and constraints. Labeling methods allow both relative and absolute quantification. The labeling can be performed at the protein level or at the peptide level. Labeling at the protein level offers the advantage of easy interpretation and could establish a dramatic reduction in sample complexity (in the best case: one peptide per protein). However, labeling at the peptide level offers the ability to use various peptides from the same protein for proper quantification. Also the risk of inconsistencies induced by missing a single-labeled site is reduced. It is also possible to obtain quantitative information from unlabeled samples by processing the mass spectral signal intensities (comparison of the relative ion intensities). The most commonly used methods will be described.

Labeled Methods

2D DIGE

The two-dimensional difference gel electrophoresis (2D DIGE) allows a parallel separation of proteins from up to three different batches. Separation of the proteins is based on their isoelectric point (first dimension) and on their molecular weight (second dimension). This technique starts by the labeling of the protein mixtures with one

of the three available fluorescent CyDyes (Cy2, Cy3, or Cy5). These labels bind to lysine side chains (the "minimal labeling" method) or, alternatively, to cysteines (the "saturation labeling" method). Up to three samples, each with a different fluorescent color staining, can be mixed and loaded into a single 2D gel. This approach involving the simultaneous analysis of multiple samples is known as *multiplexing* and is a general advantage of labeled methods. Scanning of the gel delivers a picture of "gel spots" with diverse locations. An internal reference is constituted of an equal mix of all the processed samples. This internal reference sample therefore contains all possible spot positions of the individual samples. This facilitates the interpretation of closely migrating gel spots. Moreover, matching of the same reference sample in different gels creates an intrinsic link between these different gel runs. Matching and quantitative analysis of the spots from scanned gel images is performed by specialized software. Here, an impressive collection of software is available, including Melanie (GeneBio, Geneva, Switzerland), DeCyder 2D or ImageMaster 2D (GE Healthcare, Chalfont St. Giles, UK), PharosFX System, PDQuest 2D (Bio-Rad, Hercules, CA), Dymension (Syngene, Cambridge, UK), Progenesis SameSpots, and Delta2D. Following the differential analysis, the identification of the content of each gel spot is based on an *in-gel* digestion and subsequent analysis in MS.

Isotope-Coded Affinity Tags (ICAT)

This is one of the first tagged methods developed for quantitative mass spectrometry [1, 2]. The original tags exist in two forms, heavy and light, and react specifically with free cysteine residues. The tags have exactly the same chemical composition but differ in mass because of the presence of eightfold deuterated (heavy tag) or non-deuterated linker groups (light tag). Labeling can be performed both at the protein level and on the peptide level. Two samples, each with a different ICAT tag, are mixed to generate a multiplexed analysis. The tags also contain biotin, which allows easy separation of the tagged cysteine containing peptides by (strepta) avidin beads. ICAT offers the advantage that after digestion, only the peptide (or peptides) with the specific label is required for the quantification. This can strongly simplify the MS analysis and subsequent data processing.

As stable isotope labels should in principle affect only the mass, the biophysical and chemical properties of peptides and proteins should not be affected. Therefore, the heavy and light peptides co-elute from the LC column at the same retention time. The heavy stable isotope leads to a mass shift in the mass spectrum. The presence of both heavy and light tags results in the appearance of peak pairs, which can be compared to calculate the difference in abundance between both samples.

As the number of cysteine residues in proteins is restricted, a huge reduction in complexity of the sample can be obtained. Obvious disadvantages are that the labeling efficiency is not always optimal and that some proteins (about 10%) even do not contain cysteine residues. Additionally, the biotin tag is not small and increases the complexity of fragmentation spectra, making peptide identification more tricky.

Moreover, the deuterium atoms that are associated with the tag may lead to a shift between the light and heavy peptides in reversed-phase chromatography [3]. The method has been improved by the substitution of a cleavable and co-eluting tag [4, 5].

Isotope-Coded Protein Labeling (ICPL)

This method uses similar principle (isotope-coded tags with the same chemical composition) as ICAT, but now free lysine side chains and free N-termini are labeled. Because there are significantly more free amino groups available than free cysteine residues, the level of labeling is increased significantly. ICPL allows the simultaneous comparison of up to four experimental conditions in a single experiment [6]. Labeling can be performed at both the protein level (before digestion) and the peptide level (after digestion).

Stable Isotope Labeling by Amino Acids in Cell Culture (SILAC)

An interesting protein labeling method involves the manipulation of the culture medium to ensure that newly synthesized proteins are carrying an isotopic label. As the stable isotopes are incorporated into metabolic products (proteins), this approach is known as in vivo labeling or *metabolic* labeling. Application of this method to allow quantitative proteomics was originally reported by Oda et al. [7] in growing yeast cultures, demonstrating the inclusion of ^{15}N atoms in all amino acids by adding ^{15}N-labeled ammonium persulfate as the only nitrogen source in the culture medium. The method was further developed in 2002 by the lab of Matthias Mann [8], to create a stable isotope labeling by adding amino acids in cell culture (SILAC). In stable isotope labeling by amino acids in cell culture (SILAC), cell cultures are incubated with essential amino acids (lysine, arginine) containing heavy stable isotopes. During cell growth, those amino acids will be integrated into proteins, resulting in the integration of the labeled amino acids in the whole cell proteome.

The labeling of lysine and arginine is highly interesting because trypsin, the predominant enzyme used for protein digestion in MS analysis, cleaves at the C-terminus of lysine and arginine. Therefore, in a SILAC experiment, all tryptic peptides, with exception of the C-terminal peptide, have at least one labeled amino acid. When analyzed, this will result in a shift in the masses of the digested peptides. When the labeled samples are mixed together with the non-labeled samples, peptides will be represented by peak pairs. The mass difference between those peaks is dependent on the number and nature of the labeled amino acids. More recently, SILAC has been applied in global proteome studies [9], for functional proteomics assays, as well as for the study of post-translational modifications [10, 11]. SILAC is currently the most common approach for in vivo isotopic labeling, but is considered as an expensive and time-consuming method, with an efficiency that was reaching only 70% in plants [12],

which is not sufficient in many other proteomic studies. Moreover, it is not always suitable, in terms of use and ethics, to label the tissues in a living organism, meaning that the development of alternative chemical and enzymatic methods is also useful.

TMT and iTRAQ Isobaric Labeling

The isobaric tag for relative and absolute quantitation (iTRAQ) [13] and tandem mass tag (TMT) [14] technologies have been developed as an alternative to standard isotope-coded labeling especially to enhance the degree of multiplexing. Unlike isotopic tags, isobaric tags not only have identical chemical properties but also identical masses, resulting in perfect co-elution of heavy and light tagged peptides [15, 16]. Both TMT and iTRAQ labeling are commonly performed at the peptide level and create a covalent labeling of the N-terminus and side chain amines of peptides. The labeled peptides produce only a single peak during liquid chromatography, even when two or more samples are mixed. After fragmentation of the labeled peptide by collision-induced dissociation (CID), the specific mass tag becomes visible as one of the fragments. Therefore, this type of quantitative proteomic analysis essentially requires MS/MS. Isobaric labeling allows superior multiplexing (four, six, or even eight labels). Isobaric mass tagging has also been adapted for use with protein labeling (similar to ICPL).

Chemical Labeling

All kinds of custom chemical labeling have been described. The label is introduced into proteins or peptides by a chemical reaction, for instance, with amine groups or sulfhydryl groups. Esterification or acetylation of amino acid residues also has been applied, as well as dimethylation of the primary amines of digested peptides with isotopomeric dimethyl labels.

Enzymatic Labeling

Another labeling method involves the creation of newly formed C-termini upon trypsin digestion [17]. By digestion in heavy water ($H_2{}^{18}O$), the new C-termini will carry the heavy ^{18}O label. This method allows the comparison of two conditions in parallel (normal versus heavy C-terminus) and is cheap. Unfortunately, the label is not stable and can be lost by incubation in normal water.

Label-Free Methods

The label-free methods do not use any labeling step and are therefore very attractive because of their simplicity. In addition, problems related to incomplete labeling are also avoided. However, the data processing of whole proteomic datasets is much less straightforward, and usually the threshold to identify differences is higher than what is obtained with labeling-based methods. Therefore, if the focus is set on only tiny differences (e.g., less than 35%) in protein concentration, labeling-based methods are definitely preferred. The ease of use and the low cost compared to other quantitative proteomic approaches have established the label-free quantification strategies as the most popular methods in large-scale sample experiments such as clinical screenings or biomarker discovery experiments.

Unlike other quantitation methods, label-free samples are not multiplexed. Each sample is analyzed separately. Therefore, label-free quantitation experiments need to be more carefully controlled than stable isotope methods to account for any experimental variations. Protein quantitation is performed using either ion peak intensity or spectral counting.

SELDI-TOF MS

The surface-enhanced laser desorption ionization (SELDI) time-of-flight mass spectrometry has been applied in the past in various label-free proteomics studies. This method is rather unique as the differential analysis is performed by comparing the signal intensities from proteins and not from peptides. This technology uses special matrix-assisted laser desorption ionization (MALDI) target plates, so-called ProteinChip Arrays, which have spots with particular chromatographic surfaces (hydrophobic, cationic, anionic, metal ion presenting, or hydrophilic), allowing an on-chip purification of the sample. Also pre-activated ProteinChip Arrays are available for the coupling of diverse capture molecules (proteins such as antibodies or receptors, DNA, or RNA) prior to sample loading. The technology was originally produced by Ciphergen Biosystems Inc. (Fremont, CA, USA), later hosted by Bio-Rad, but currently this technology is no longer available, because of various limitations.

Multidimensional Protein Identification Technology (MudPIT)

This method involves a digestion of the sample and subsequent analysis by a multidimensional liquid chromatography (more than one LC)-MS setup. Multidimensional protein identification technology (MudPIT) was originally described in 2001 by the group of Yates [18], with a first chromatography dimension consisting of a strong cation exchanger (SCX), and the second dimension consists of a reversed-phase

chromatography. This online two-dimensional high-performance liquid chromatography (HPLC) can separate well-complex peptides, and the output of the second liquid chromatography (LC) is directly connected to the mass spectrometer. Recent method developments in peptide separation are using alternative separation strategies to SCX to improve peak separation and hence increase peptide identifications for MudPIT. A promising method is the use of "high pH-reversed-phase" separation as the first dimension. The use of this method increases peptide identifications by a factor of two when compared to similar MudPIT runs.

The use of "virtual 2D mapping," with the elution time from the column in one axis and the measured MS ions in the other axis, has been proven to be very powerful in differential analysis and quantification of the obtained results. The developed software tools (e.g., DeCyder MS, Progenesis, etc.) could build upon the large expertise generated from the data processing of 2D gels.

Current mass spectrometers demonstrate a huge improvement in resolution, accuracy, and speed, and some of them offer an additional separation such as ion mobility. Together with the recent developments at the LC level (nanoLC using ultra-performance liquid chromatography (UPLC) or ultrahigh-performance liquid chromatography (UHPLC), "chip-based" microfluidic systems, etc.), it should be clear that various new LC-MS or LC-LC-MS workflows are under investigation.

Liquid Chromatography Coupled to Fourier Transform Mass Spectrometry (FTMS)

Fourier transform mass spectrometry (FTMS) using Fourier transform ion cyclotron resonance (FTICR) or using an Orbitrap analyzer outperforms any other commonly used mass spectrometry setup in terms of resolution (separation power) and accuracy. The Orbitrap-based MS instruments are currently recognized as the standard for accurate mass and high-resolution measurements, and the Orbitrap Q Exactive combines superior dynamic range and unsurpassed sensitivity with the high-performance quadrupole precursor selection and the high-resolution, accurate-mass Orbitrap detection to deliver high performance and tremendous versatility. An Orbitrap Q Exactive mass spectrometer linked to a nanoflow liquid chromatography (nanoLC) represents a platform that not only can offer broad screening capabilities but also excels at targeted quantitation of molecules of interest (candidate biomarkers).

Proteomics in Endometriosis

Proteomics is the large-scale study of proteins, their expression, localization, functions, post-translational modification, and interactions [19]. Proteomics allows the simultaneous observation of alterations in protein expression which may be either a

precursor to or causative in disease development or consequence of the disease [20]. Endometriosis researchers found differentially expressed protein/peptides between women with and without endometriosis in blood and urine but also in eutopic and ectopic endometrium [21, 22]. However, there is a general lack of studies that focus on the validation of biomarkers which to date still no biomarker or panel of biomarkers is sufficiently validated for clinical use [22].

SELDI-TOF MS platform has been used in endometriosis. Both eutopic endometrial specimens from women with and without endometriosis [23, 24] and blood samples have been used [22]. Briefly, SELDI-TOF MS provides differential proteomic profiles in the form of mass/charge (m/z) peaks without identification of the peptides or proteins, rather a fingerprinting. Kyama and coworkers were the first to use SELDI-TOF MS for endometriosis research and found reduced expression of a protein peak in secretory-phase endometrium from women with mild endometriosis relative to controls [25]. The same group found 32 peptide peaks differentially expressed in secretory-phase endometrium from women with endometriosis ($n = 10$) compared to controls ($n = 6$) [26]. Other research groups found five differentially expressed peptide peaks (5.385 m/z, 5.425 m/z, 5.891 m/z, 6.448 m/z, and 6.898 m/z) that collectively showed 91.7% sensitivity and 90% specificity in the diagnosis of endometriosis [24]. A panel of three differentially expressed peptide peaks (16.069 m/z, 15.334 m/z, and 15.128 m/z) diagnosed endometriosis with 87.5% sensitivity and 86.2% specificity [27].

In an exploratory study, a panel of four mass peaks (two upregulated, 90.675 kd and 35.956 kd, and two downregulated, 1.9 kd and 2.5 kd) allowed the identification of endometriosis with maximal sensitivity (100%) and specificity (100%) [28, 29]. The 90.675 kd and 35.956 kd mass peaks were identified as T-plastin and annexin V proteins, respectively [28, 29]. Annexin has a role in proliferation and/or cell mobility, has metastatic potential, and may promote the pathogenesis of endometriosis by stimulating early invasion of endometrial cells into the mesothelium after initial attachment to the peritoneal T-plastin plays a role in cellular motility, formation of the actin bundles required for cell locomotion, and maintenance of the cellular architecture [29]. The same group described a panel of differentially expressed peptide peaks (2072 m/z, 2973 m/z, 3623 m/z, 3680 m/z, and 21,133 m/z) in the early secretory endometrial proteome of women with versus without endometriosis as diagnostic of endometriosis with 91% sensitivity and 80% specificity [23].

In peripheral blood, SELDI-TOF MS and MALDI-TOF MS investigations have also shown differentially expressed protein and peptides in women and without endometriosis [30–40]. The largest study made an effort to identify the protein/peptide peaks with altered levels after analysis of 254 plasma samples from women with ($n = 165$) and without ($n = 89$) endometriosis [30]. Ultrasonography-negative endometriosis was best predicted (sensitivity 88%, specificity 84%) using a model based on five protein/peptide peaks (2.058 m/z, 2456 m/z, 3.883 m/z, 14.694 m/z, and 42.065 m/z) in plasma samples obtained during the menstrual phase [29, 30]. 2189 m/z was identified as fibrinogen beta-chain and was decreased in moderate-severe women of endometriosis. Fibrinogen beta-chain has been patent for endometriosis; this group Fazleabas found decreased levels in uterine flushing of baboons

with induced endometriosis. A proteomic fingerprint model (126 endometriosis patients and 120 healthy controls), based on three peptide peaks, had 91.4% sensitivity and 95% specificity to detect endometriosis [38]. These results were validated in an independent cohort, showing a sensitivity of 89.3% and a specificity of 90% [38]. In a study by Dutta et al., using 2DE and 2D DIGE followed by MALDI analysis, 25 serum proteins were found to be differentially expressed between women with endometriosis and healthy subjects [40].

Hwang et al. used 2DE followed by MS and showed six differentially expressed plasma proteins between plasma pools of women with ($n = 15$) and without ($n = 15$) endometriosis [41]. Only haptoglobin was identified as potential biomarker using Western blotting on a subset of the individual samples [41].

Recently, one research group has reported 36 differentially expressed peptides in urine samples of women with endometriosis ($n = 60$) compared to women without endometriosis ($n = 62$) detected by MALDI-TOF MS. Using ClinProTools software, they generated an algorithm with a combination of five peptide peaks (m/z = 1433.9, 1599.4, 2085.6, 6798.0, 3217.2) [42]. Only one other group has identified six differentially expressed protein/peptides in urine of women with and without endometriosis [43]. The results were comparable between El Kasti group [43] and Wang et al. group [42]; however, both were not able to identify the protein/peptides.

Proteomics does not only imply protein/peptide differentiation but also post-translational modification. Post-translational modification occurring within cells is mainly responsible for the discrepancies noted between the genome and the expressed proteome. Currently, ~300 different types of PTM are responsible for the huge repertoire of protein origination from a small number of genes [44]. A study investigating the endometrial phosphoproteome of women with ($n = 4$) and without ($n = 4$) endometriosis showed that 516 proteins were modified at phosphorylation level during endometriosis [45]. Recent evidences have emerged that endometriosis may be an epigenetic disease [46]. Epigenetics refers to functionally relevant modifications to the genome that do not involve a change in the nucleotide sequence; this process is involved in development, homeostasis, disease, and aging and is responsible for X chromosome inactivation and genomic imprinting [46]. Histone proteins are located in the central part of chromatin, where they provide binding sites for covalent modification at their N-terminus [46]. Histone-modifying enzymes, such as HATs, HDAC, and HMTs, could affect structure of nucleosome and chromatin through modifying histone proteins posttranscriptionally, which in turn regulates gene expression pattern [46].

Future Trends

Endometriosis represents a significant global health burden, and proteomic approaches offer one avenue to discover new molecules allowing more sensitive and specific detection or diagnostic strategies [47]. To date, none of the differentially expressed protein/peptide peaks have been validated in an independent study cohort

(blinded method as to patients' disease status). Standardization is essential to overcome any pitfalls in the study design and methodology such as small sample size, lack of relevant clinical information, inconsistency in sample handling and storage, and technical control of pre-analytical sample variability [29]. The right documentations of the type of samples and highly standardized techniques for collection, processing, and storage are very important [47–49]. The depletion method is a crucial item in the design of future studies [30] to decrease the complexity of highly abundant proteins.

Many problems remain to be resolved, and while some of these are technical in nature, the most intractable ones have mainly to do with the complex and multifactorial character of the disease itself [20]. The analysis of differential protein expression in such complex biological samples requires strategies for rapid, highly reproducible, accurate, and robust protein quantitation [47] preferentially using Fourier transform mass spectrometry (FTMS).

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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).

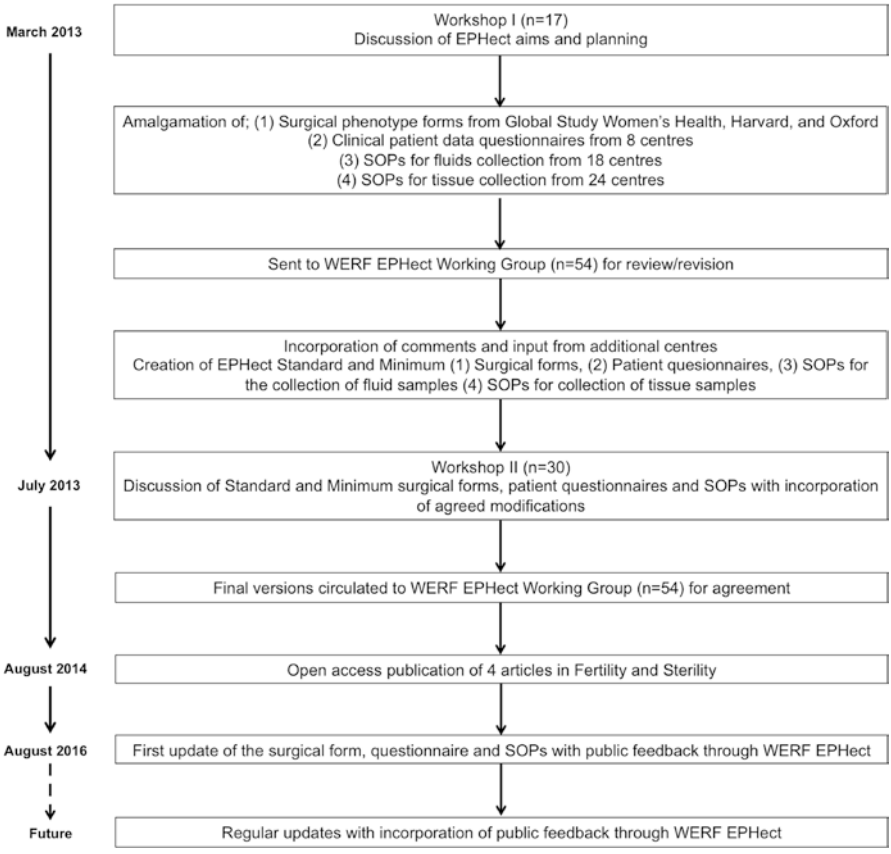

Fig. 1 Flow diagram depicting the WERF EPHect development and consensus process (Adapted from [25–28])

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

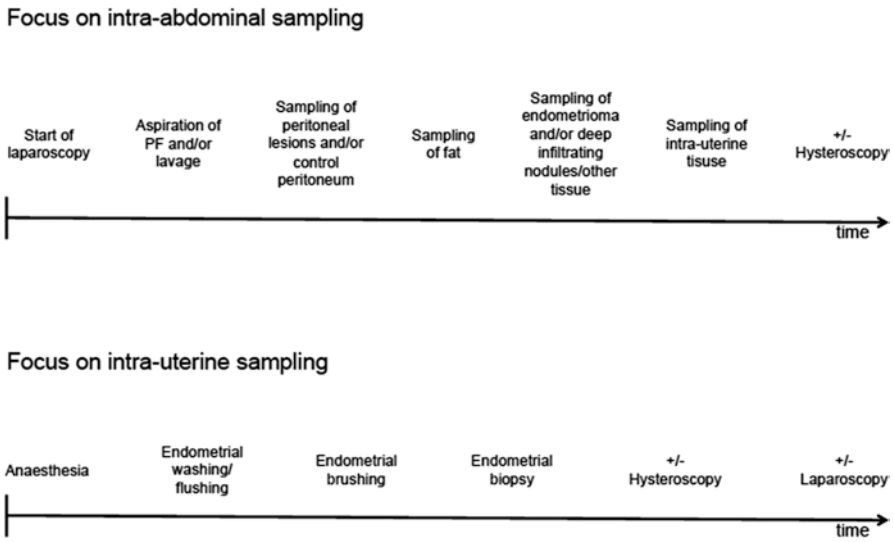

Fig. 2 Suggested timeline for biological sample collection depending on research question (From [25])

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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OMICs Studies and Endometriosis Biomarker Identification

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Abstract The fast development and capabilities of high-throughput 'omics' technologies have provided new insights into the complexity of endometriosis and enabled the identification of novel diagnostic biomarkers. In this chapter, we take a closer look at high-throughput genomics, transcriptomics, epigenomics, proteomics, and metabolomics studies applied in endometriosis research. We summarise the existing information concerning 'omics' studies applied to blood, endometrium, endometriotic lesions, and body fluids in order to describe the potential disease-specific biomarkers. Also, we discuss the importance of sample collection, proper study design, data processing, and analysis in high-throughput studies. And finally, future perspectives in endometriosis biomarker research will be provided.

Keywords Endometriosis • Epigenome • Biomarkers • Metabolome • Omics • Proteome • Systems biology • Transcriptome

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Abbreviations

aCGH	Array-based comparative genomic hybridization
CNV	Copy number variation
CpG	C-phosphate-G-site
2D-DIGE	Two-dimensional difference gel electrophoresis
ESI-MS/MS	Electrospray ionisation tandem mass spectrometry
GWAS	Genome wide association study
H-NMR spectroscopy	Proton nuclear magnetic resonance spectroscopy
LCM	Laser capture microdissection
lncRNA	Long non-coding RNA
MALDI-TOF-MS	Matrix-assisted laser desorption/ionisation time-of-flight mass spectrometry
miRNome	Full spectrum of expressed miRNAs
NMR	Nuclear magnetic resonance spectroscopy
SCNA	Somatic copy number alteration
SELDI-TOF-MS	Surface-enhanced laser desorption/ionisation time-of-flight mass spectrometry
SNP	Single nucleotide polymorphism
WERF EPHect	World Endometriosis Research Foundation Endometriosis Phenome and Biobanking Harmonisation Project

Introduction

Conventional methods have not been very successful in endometriosis-specific biomarker discovery, and to date there are no reliable non-invasive or minimally invasive diagnostic markers for endometriosis. Therefore, there is a considerable need for non-invasive biomarkers, because due to the non-specific symptoms, the average delay between the onset of symptoms and the surgical diagnosis is almost 7 years [1]. The delayed diagnosis may in turn lead to more severe complications and is associated with remarkable healthcare costs [2]. Objective and reliable non-invasive diagnostic biomarkers would not only avoid the unnecessary laparoscopy in suspicious cases but would also make it possible to get the diagnosis of endometriosis earlier and thus provide an easy strategy for monitoring the disease treatment efficacy and recurrence [3]. However, despite extensive research in this field during the past 10 years, there are still no reliable non-invasive diagnostic markers for endometriosis [4, 5], and numerous women with nonspecific complaints, such as infertility and pelvic pain, undergo diagnostic laparoscopy. Thus, ‘omics’-level studies using both easily assessable materials like blood, urine, and menstrual blood but also endometrium and endometriotic lesions are one of the top research priorities in the field.

The high-throughput techniques provide massive data from the genome (variability in DNA sequence in the genome, i.e. genomics), epigenome (epigenetic modifications of DNA, i.e. epigenomics), transcriptome (variability in composition and abundance of mRNA and miRNA levels, i.e. transcriptomics), proteome (variability in composition and abundance of the proteins, i.e. proteomics), and metabolome (variability in composition and abundance of metabolites, i.e. metabolomics/metabonomics). The major advantage of 'omics' studies is that the data can be collected without existing hypotheses, and a primary research question is not always needed (first experiment-then-hypothesise approach) [6]. This could be particularly useful when studying complex diseases with unknown pathogenesis, such as endometriosis. There are still many missing pieces in the puzzle of endometriosis, and the new 'omics' studies promise to add new biological knowledge transferrable into the development of disease-specific biomarkers. The considerable increase (15 publication in 1999–2006, 104 publication in 2007–2016, altogether 118 studies) in 'omics' research is a definite sign that the 'omics' revolution in endometriosis is actively ongoing.

In this chapter, we take a closer look at the high-throughput studies applied in endometriosis research, namely, genomics, transcriptomics, epigenomics, proteomics, and metabolomics (Fig. 1). We summarise the existing information concerning endometrium, endometriotic lesions, blood, and body fluids in order to describe the potential disease-specific biomarkers. Also, future perspectives of single-cell 'omics' in endometriosis biomarker research will be provided. And finally, we discuss the importance of sample collection and proper study design in high-throughput studies.

Search for Endometriosis Biomarkers: 'Omics' Studies and Endometrium

Endometrium is not just a uniform tissue that undergoes cyclical changes under the influence of endogenous hormones, cytokines, and chemokines but an assortment of different cells, each with their own special functions responsible for tissue differentiation, desquamation, and regeneration. It is evident that eutopic endometrium of women with endometriosis functions normally and has almost comparable responsiveness to steroid hormones; however, there is evidence from epigenomic, transcriptomic, and proteomic studies that endometrial tissue from patients with endometriosis and healthy women is differently regulated at the molecular level. Therefore, understanding the complex mechanisms controlling the changes within the endometrium is crucial to find endometrial biomarkers for endometriosis.

Genomic studies focusing only on eutopic endometria of endometriosis patients have not been very popular, and to date, only two studies have investigated somatic DNA mutations in endometrium (Table 1). Guo et al. found a number of individual chromosomal losses and gains in laser capture microdissection (LCM)-harvested

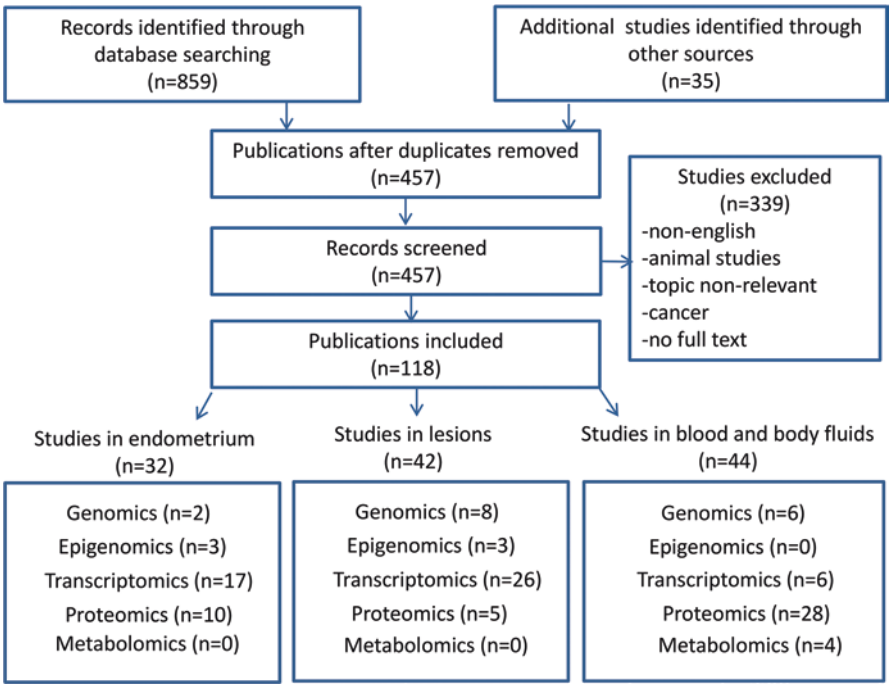

Fig. 1 'Omics' publications in endometriosis studies. Literature search was performed in PubMed up to December 2016. Only publications that were in English were considered. The keyword 'endometriosis' was one-by-one searched with terms: 'endometrium + microarray', 'miRNA + microarray', 'sequencing', 'microarray', 'gene expression + microarray', 'exome sequencing', 'GWAS', 'CNV', 'genomics', 'proteomics', 'metabolomics', 'DNA methylation + microarray', 'DNA alterations + microarray', and 'proteome'. Some of the eligible studies were identified using the reference list of appropriate review articles. In total 118 'omics' studies were included into this review chapter

endometrial epithelial cells and hypothesised that these genomic alterations could be the proximate cause of endometriosis [7]. Li et al. conducted whole-exome sequencing of blood DNA and LCM-harvested endometrial cells from eutopic and ectopic endometria of 16 endometriosis patients and eutopic endometria of 5 healthy women [8]. They found that DNA originating from healthy endometria contains thousands of somatic mutations that are absent in blood DNA. Furthermore, the general somatic mutation spectrum in endometria of women with and without endometriosis was very similar and authors proposed that most of the mutations are probably benign and irrelevant to endometriosis pathogenesis [8].

Aberrant DNA methylation is shown to contribute to many human diseases, and there is accumulating data from DNA methylation studies that methylation alterations in certain genes could contribute to the pathogenesis of endometriosis (reviewed [9]). So far, three studies have applied genome-wide microarray-based DNA methylation analysis to eutopic endometria of endometriosis patients [10–12] (Table 1).

Table 1 'Omics' studies in endometriosis

	Patients[a] (*n*)	Controls[a] (*n*)	Main findings	Reference
Endometrium				
Genome studies	21	9	Gains: +3p, +10q, +13q; losses: −1p, −3p, −4p, −22q 724 mutated genes	[7, 8]
Epigenome studies	55	46	No common genes	[10–12]
Transcriptome studies	330	203	Differences in PI3K/AKT, JAK/STAT, SPK/JNK, and MAPK, p53, adherens junction, calcium signalling, EGF/PGF/DGF, endothelial biology, protein synthesis, cell division, integrin-mediated cell adhesion, RAS/RAF signalling, decidualization, cellular adhesion, cytokine-cytokine receptor interaction, apoptosis, complement pathway	[13–25, 28–32]
			Two miRNAs reported at least in two studies	
Proteome studies	100	97	Vimentin, peroxiredoxin, HSP70, HSP90, annexins, actins, and 14-3-3 family proteins	[13, 35, 37–44]
Lesions				
Genome studies	130	9	Frequent SCNAs: Gains: 1p, 3p, 6q, 17q, and Xq; Losses: 1p, 5p, and 6q	[46–52]
Epigenome studies	24	27	*HOXD10*	[54–56]
Transcriptome studies	281	96	Differences in expression of genes involved in organ development; metabolism; action of prostaglandins and glucocorticoids; complement, RAS, MAPK, and PI3K signalling; cytokine-cytokine receptor interaction; cellular adhesion; immune cell recruitment; apoptosis; cell signalling; T-cell cytotoxicity and regulation of inflammatory responses pathways; miR-200 family (epithelial-mesenchymal transition)	[23, 30–32, 51, 57–71, 73–78]
Proteome studies	35	19	Glycolysis and oxidative respiration, transforming growth factor β-1, calponin-1 and emilin-1, SM-22α and Rab37, Rho-GDIα, haptoglobin, transgelin, smooth muscle actin-binding protein	[80–84]

(continued)

Table 1 (continued)

	Patients[a] (*n*)	Controls[a] (*n*)	Main findings	Reference
Blood and body fluids				
Genome studies	14688 2226	161694 18024	11 significant SNPs 9 CNVs	[87–90, 95, 101, 102]
Transcriptome studies	79	69	No common miRNAs, 12 miRNAs reported at least in two studies	[105–110]
Proteome studies	1970	1104	Serum/plasma: HP and A1BG PF: α1-antitrypsin, α-1b-glycoprotein, S100-A8, serotransferrin, acute phase proteins (haptoglobin and SERPINA1) Menstrual blood: RMP2, UCH-L1, MYL9 Urine: cytokeratin-19, VDBP EF: proteins involved in cell signalling, cell death, and cell movement processes	[37, 44, 111–137]
Metabolome studies	119	114	SMOH C16:1, ratio (PCaa C36:2/PCae C34:2), 2-methoxyestradiol, 2-methoxyestrone, dehydroepiandrostion, androstenedione, and cholesterol	[140–143]

[a]The total number of patients or controls in this type of study. *PF* peritoneal fluid, *EF* endometrial fluid

The study by Naqvi et al. described several aberrantly methylated and expressed genes, among them *MGMT*, *DUSP22*, *CDCA2*, *ID2*, *TNFRSF1B*, *ZNF681*, and *IGSF21* have previously not been associated with endometriosis [10]. Although several formerly known genes with altered methylation levels, including *MAFB*, *HOXD10*, and *HOXD11*, were highlighted, alterations in DNA methylation levels in other genes (*PR-B*, *CYP19A1*, *SF1*, *COX2*, and *ER-β*) previously associated with endometriosis were not confirmed. The study by Saare et al. showed that the endometrial DNA methylation profiles were highly similar between endometria of patients and controls but largely influenced by the menstrual cycle phases [11]. Authors suggested that DNA methylation differences are likely not the main reason for endometriosis development, but it is crucially important to take into account the normal epigenetic changes across the menstrual cycle when looking for disease-specific methylation differences in endometrium. A subsequent study by Houshdaran et al. compared endometrial DNA methylation patterns and associated gene expression levels in endometriosis patients and healthy controls across the menstrual cycle and found a small number of differentially methylated loci between the patients and controls [12]. The differences in endometrial DNA methylome were most contrasting between the patients and controls in the mid-secretory phase (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).

Interestingly, there were no overlapping differentially methylated genes in all three genome-wide studies [10–12]. Based on these results, it can be proposed that the normal physiological fluctuations during the menstrual cycle may have larger impact on endometrial DNA methylation signature than disease/non-disease status, and thus, the DNA methylation changes in endometria of patients is probably not the primary cause for endometriosis development.

Several transcriptome studies have used mRNA microarray technology to resolve the question whether there are any differences between endometria of patients with endometriosis and healthy women [13–25] (Table 1). While a majority of these studies have yielded numerous candidate genes, the amount of genes which have consistently been shown as up- or downregulated has remained small. Aghajanova and Giudice provided evidence that also the endometria from patients with different endometriosis stages have differences on the molecular level [18]. Further, the authors proposed that the influence of menstrual cycle phase on endometrial transcriptome could be larger than the presence or absence of endometriosis. Still, dysregulation of progesterone and/or cyclic adenosine monophosphate (cAMP)-regulated genes and genes related to thyroid hormone action and metabolism between endometria of patients with different endometriosis stages and menstrual cycle phases was found. Also, upregulation of epidermal growth factor receptor (*EGFR*) and extracellular matrix proteoglycan versican (*VCAN*) during the early secretory phase was found in severe versus mild disease [18]. The pathway analysis of differently expressed genes in endometria of patients with severe endometriosis exhibited dysregulation of PI3K/AKT, JAK/STAT, SPK/JNK, and MAPK pathways that have been associated with endometriosis pathogenesis in several studies [26]. The study conducted by Tamaresis et al., comparing endometria of patients and controls, found 18 upregulated and 11 downregulated genes in all three studied menstrual phases, and also a number of genes were dysregulated in patients with different stages of the disease [19]. They used gene expression data of 148 women to develop a molecular classifier that distinguishes endometria of women with and without endometriosis and found that the best performing classifiers, enabling identification of endometriosis with 90–100% accuracy, were mostly menstrual phase specific and utilised relatively few genes to determine the presence and severity of the disease. Multiple pathways were found to be activated in the proliferative and early secretory phase endometrium (JAK/STAT, EGF/PGF/DGF, PI3K-AKT signalling, p53 signalling, integrin-mediated cell adhesion) of women with moderate-severe endometriosis compared to minimal-mild endometriosis [19], and this was in good concordance with the previous results [18]. Dysregulation of the RAS/RAF/MAPK and PI3 kinase signalling pathway genes, which participate in a wide variety of cellular functions and cell survival, is identified in several studies [18–20, 25], referring to a link between these pathways and disease pathogenesis. Ahn et al. noticed that based on the unsupervised hierarchic clustering analysis, the overall gene expression signature of endometria from patients and controls was similar [23]. Still, 91 differentially expressed genes involved in regulation of decidualization, cellular adhesion, cytokine-cytokine receptor interaction, apoptosis, and complement pathway were found. In the latest study by Zhou et al., mid-secretory endometria from

patients and controls were analysed and 357 differentially expressed mRNAs were found to be involved in signalling pathways such as the JNK/MAPK, PI3K-AKT, p53, adherens junction, and calcium signalling pathway [20]. In addition to studies reporting distinct endometrial molecular signatures of endometriosis patients, there are also evidence that endometrial receptivity gene signature during the implantation window is similar in patients with endometriosis and healthy women [22, 27].

Several microarray-based microRNA (miRNA) studies concentrating on eutopic endometria have been performed [28–32]. Burney et al. studied eutopic endometria from patients and controls to reveal a disease-specific endometrial miRNA signature [28]. They found six downregulated miRNAs from miR-9 and miR-34 families in eutopic endometria of endometriosis patients and suggested that downregulation of miR-34 family could be involved in maintaining the molecular fingerprint in proliferative endometrium and mediate the delayed proliferative to secretory transition observed in women with moderate-severe endometriosis [28]. A following study by Laudanski et al. reported a lower expression of miR-483-5p and miR-629* in the eutopic endometrium of women with advanced ovarian endometriosis compared to controls [29]. They suggested that expression changes of these miRNAs are a consequence of an early defect in the physiological activity of the proliferative endometrium, ultimately resulting in the overgrowth of this tissue outside the uterus [29]. Subsequently, Laudanski et al. utilised a more comprehensive array and reported the presence of 136 upregulated miRNAs in the eutopic endometrium of patients with endometriosis compared with the healthy women [30]. However, after validation, only three out of 11 validated miRNAs revealed borderline significance. In the study by Braza-Boils et al., both eutopic endometria from patients and controls and endometriotic tissues were studied, and only five miRNAs were found to be differentially expressed in eutopic endometria of endometriosis patients compared to healthy endometrium [31]. Thirty-six downregulated miRNAs in endometria of patients were also reported by Shi et al. [32]. However, the comparison of all results from aforementioned miRNA studies showed a minute overlap, and only two miRNAs (miR-9* [28, 32] and miR-636 [31, 32]) were reported in at least two studies. Therefore, as different miRNA studies have reported different candidate miRNAs, the potential application of endometrial miRNAs as endometriosis biomarkers is still limited. Clearly, our knowledge about the endometrial miRNome and its physiological and pathophysiological significance in association with endometriosis is scarce and remains to be unravelled.

The functional interpretation and understanding of the proteome is one of the current challenges in biology due to the presence of sequence variations, alternative splicing, and epigenetic and post-translation modifications [33, 34]. The complexity of the proteome is illustrated by the fact that there is a poor correlation between the transcript levels and the abundance of the corresponding proteins [35, 36]. Proteomic research in endometriosis is currently a ‘hot topic’, and a number of endometrial proteome studies have been performed in endometriosis patients [13, 35, 37–44] (Table 1). A long list of potential disease-related proteins has been proposed but only a few of them, like vimentin, peroxiredoxin, HSP70, HSP90, annexins, actins, and 14-3-3 family proteins (phosphoserine- or phosphothreonine-binding proteins),

are consistently identified as differentially expressed in patients in at least three different studies. A recent excellent review by Siva et al. summarises the current situation in the proteomic research field—so far no clear biomarker or therapeutic targets have been discovered [45]. Nevertheless, as the protein synthesis is the final result of the gene expression and is directly linked to the phenotype, the endometrial proteome studies do hold a great promise for future biomarker discovery.

Taken together, the large-scale 'omics' studies have provided clear evidence that the endometrial genome, epigenome, transcriptome, and proteome are differently regulated in endometriosis. Although the concordance between different 'omics' studies has been moderate, some potential biomarkers such as miR-9 and miR-636 family; disease-related pathways PI3K/AKT, JAK/STAT, SPK/JNK, and MAPK from transcriptome studies; and proteins like vimentin, peroxiredoxin, HSP70, HSP90, annexins, actins, and 14-3-3 family members from proteome studies have been proposed.

'Omics' Studies of Endometriotic Lesions and Possible Biomarkers

When it comes to biomarkers research, endometriotic lesions are a less-favoured study object than endometrial biopsies, as lesions do not provide direct non-invasive or minimally invasive biomarkers for clinical use. Nevertheless, the studies using lesions are crucial for detecting molecular alterations involved in the disease development and pathogenesis and thereby provide valuable information for biomarker research.

Microarrays and single nucleotide polymorphism (SNP) genotyping technologies together with recent advances in high-throughput sequencing have led to a rapid progress in genomic studies in endometriosis and provided new evidence about the genetic background of the disease. However, genome-wide studies have provided no clear consensus about the somatic DNA alterations either in endometriotic lesions and/or eutopic endometria. A number of studies have reported chromosomal alterations, more frequently gains in chromosomes 1p, 3p, 6q, 17q, and Xq and losses in chromosomes 1p, 5p, and 6q [46–50], while other studies have found no chromosomal aberrations in ectopic endometrial tissue or eutopic endometrium [51, 52], thus raising a question about the relevance of DNA genomic imbalance in the pathogenesis of endometriosis (Table 1). Saare et al. used SNP microarrays instead of traditional array comparative genomic hybridization (aCGH)] to compare the same patients' blood, endometria, and LCM-harvested cells of endometriotic lesions and found no evidence of disease-specific somatic DNA copy number alterations (SCNAs) [52]. The authors suggested that some SCNAs identified in previous studies may be related to the detection methodology (CGH or array-CGH) as it has been shown that some G-C-rich chromosomal regions (1p and 16p and chromosomes 19 and 22) tend to give false-positive results [50, 53].

The identification of epigenetic biomarkers for endometriosis diagnostics is definitely an emerging, challenging, and still largely uncovered field of investigation. In the recent years, researchers have turned their major interest from transcriptome to epigenome, and a few large-scale epigenome studies, describing the lesion-specific DNA methylation profiles, have been performed (Table 1). The genome-wide DNA methylation profiles of endometriotic lesions or stromal cells originating from lesions have been described in three studies [54–56]. Borghese et al. published the first study describing the global DNA methylation profile of different endometriotic lesions, including ovarian endometriomas, deep infiltrating endometriosis, and superficial endometriosis, and showed that global methylation pattern was similar in different lesion types and eutopic endometria [55]. When global methylation data of eutopic endometria was compared to ovarian endometriomas in combination with gene expression data, 35 genes were found to share alterations both in methylation and expression patterns [55]. Specific regions were consistently hypermethylated (or hypomethylated) in all subtypes of the disease, and other regions were strictly altered in one endometriosis type only, and variation in methylation was more likely to occur at discreet loci across the genome. The later study by Dyson et al. found more than four thousand differentially methylated CpGs when stromal cells from eutopic endometria were compared to stromal cells from endometriomas, and the authors concluded that endometriotic cells possess a unique epigenetic fingerprint [54]. The analysis of differentially methylated and expressed genes identified 403 genes that were aberrantly methylated and differentially expressed in endometriosis, among them are genes from the *HOXA* cluster, *ESR1*, *NR5A1*, and *GATA* family transcription factors [54]. Although a different study design (entire lesions vs. cultivated stromal cells) and platforms were applied to interrogate DNA methylation, both investigations [54, 55] reported different methylation of *ADAP1*, *HPCAL1*, *PRKAG2*, *PRKCZ*, *RIPK1*, *SEC61A1*, *ZNF22*, and *HOXD10* genes. Most recent study by Yamagata et al. analysed stromal cell cultures from endometriomas and eutopic endometria of patients and controls and found that methylation profiles of eutopic endometria were very similar but significantly different from stromal cells originating from endometriomas [56]. The genes with altered methylation in endometriomas were related to signal transduction, molecular functions of receptors and signalling molecules, and cytokine-cytokine receptor interactions and development. Comparison of datasets from Yamagata et al. and Dyson et al. revealed four overlapping genes: *HOXD10*, *BST2*, *GATA4*, and *TCF21*, but when all three DNA methylation studies were compared to each other, only *HOXD10* was seen to be differentially methylated.

The first transcriptome studies in endometriosis applying microarray technology were conducted already in 2002, and since then, many studies have been carried out to reveal the specific gene expression profile of endometriotic lesions. Transcriptome studies in lesions can be divided into two groups—studies performed in 2002–2007 [57–65] that used less comprehensive microarrays [up to 23 thousand (23K) probes] and studies conducted in the recent years using advanced large-scale microarrays covering 44K or 60K probes [23, 30, 51, 66–71] (Table 1). Although the list of can-

didates in each study contains a remarkable number of dysregulated genes in ectopic endometrial tissue compared to eutopic endometria, there is little concordance in the reported genes between studies. However, altered expression of genes belonging to RAS, MAPK, and PI3K signalling pathways was proposed in several studies (reviewed in [26]). Khan et al. found 50 differently expressed genes associated with immunological, neurocrine, and endocrine functions and gynaecological cancers (*CHEK1*, *ERBB* family, laminin gamma, and *Ki-67*), but there was no overt oncogenic potential in endometriotic tissue [66]. Also, they reported a list of 28 novel genes that were not previously associated with endometriosis, representing potential markers for ovarian endometriosis. The following studies by Monsivais et al., Crispi et al., and Suryawanshi et al. found many dysregulated genes that belong mostly to tissue and organ development pathways [68], pathways regulating metabolism and action of prostaglandins and glucocorticoids [72], and complement pathway [24]. Sun et al. used a microarray comprising of probes for long non-coding RNAs (lncRNA) and mRNAs and found hundreds of dysregulated lncRNAs and thousands of mRNA transcripts in ectopic endometrial tissues compared to paired eutopic endometrial tissues [67]. Authors proposed that many dysregulated lncRNAs may participate in biological pathways related to endometriosis through cis- and trans-regulation of target protein-coding genes. In the latest study by Ahn et al., a large number of differentially expressed genes involved in cytokine-cytokine receptor interaction, cellular adhesion, immune cell recruitment, apoptosis, cell signalling, T-cell cytotoxicity, and regulation of inflammatory responses were found [23].

To date, eight high-throughput miRNA studies describing the miRNome of the whole endometriotic lesion biopsies or cultured stromal cells from lesions have been performed [31, 32, 73–78] (Table 1). Each study has identified a subset of miRNAs that has been differently expressed in ectopic lesions compared to eutopic endometria. As there is a large variability between studies in the terms of design, analysis methods, and selection of controls, the concordance has been moderate, and only 22% of reported miRNAs are consistent between studies [79]. In addition to microarrays, next-generation miRNA sequencing technology has also been applied in endometriosis studies [73, 76]. Hawkins et al. compared specimens from endometrioma and normal endometrium and found several miRNAs that were upregulated (miR-29c, miR-100, miR-193a-5p, miR-202, miR-485-3p, miR-509-3-5p, miR-708, and miR-720) or downregulated (miR-10a, miR-34c-5p, miR-141, miR-200a/b/c, miR-203, miR-375, miR-429, miR-449b, miR-504, and miR-873) in endometriomas [73]. The following study by Saare et al. investigated paired samples of peritoneal endometriotic lesions and matched healthy surrounding tissues together with eutopic endometria of the same patients and found five miRNAs (miR-34c-5p, miR-449a, miR-200a, miR-200b, and miR-141) that were significantly overexpressed in lesions compared to healthy surrounding tissues [76]. Although majority of these miRNAs were reported to be associated with endometriosis pathogenesis in Hawkins et al. [73] and Ohlsson Teague et al. [75] studies, Saare et al. [76] concluded that these miRNAs rather reflect the presence of endometrial cells in the peritoneal tissue than are associated with pathologic events.

Furthermore, the authors suggested that the miRNA profile of peritoneal endometriotic lesions is largely masked by the surrounding peritoneal tissue present in biopsy samples, challenging the discovery of an accurate lesion-specific miRNA profile [76]. Still, it should be pointed out that according to the results of all these miRNome studies, there was only one single miRNA (miR-200b) that was differentially expressed in all six studies in whole lesions [32, 73–77] but not in endometriotic stromal cells [78]. miR-200b, member of the miR-200 family, could be an attractive molecular marker that can be easily linked to disease pathogenesis because of its important role in cell migration and epithelial-mesenchymal transition (EMT). It could be proposed that altered expression of miR-200b in lesions changes the well-balanced network of EMT and leads the endometrial epithelial cells to acquire mesenchymal phenotype with higher migratory capacity.

To summarise, transcriptome studies of endometriotic lesions have already provided some clues about the pathogenesis of endometriosis, though the concern still remains about using whole-tissue biopsies instead of pure populations of endometrial epithelial and stromal cells from lesions to reveal transcriptome changes inside the lesion.

Only a few studies on endometriotic tissue proteomics have been conducted so far [80–84] (Table 1). However, it could be hypothesised that if disease-specific proteins with high concentration in affected tissues exist and are secreted from lesions into the blood stream, they could also be monitored in the body fluids [81] and thereby could offer potential for discovery of non-invasive markers. The results of proteome studies have shown that some proteins are differentially expressed (e.g. SM-22α and Rab37), modified (e.g. haptoglobin and Rho-GDIα,), or localised (e.g. haptoglobin) in endometriotic lesions compared to eutopic endometria of patients or healthy women [80]. Also, significant increase in transforming growth factor β-1, calponin-1, and emilin-1 [81] in ovarian endometriomas has been reported. In the recent study by Kasvandik et al., metabolic reprogramming of ectopic endometrial stromal cells with extensive upregulation of glycolysis and downregulation of oxidative respiration was noticed [82].

The above-discussed examples from genomic, epigenomic, transcriptomic, and proteomic levels have provided inconsistent evidence about the possible molecular changes occurring in the endometriotic lesions. The genomic studies in endometriotic lesions have reported chromosomal alterations more frequently in chromosomes 1p, 3p, 5p, 6q, 17q, and Xq; epigenome studies have provided evidence on altered DNA methylation in *HOXD10*; transcriptome studies have found altered expression of genes belonging to RAS, MAPK, and PI3K signalling pathways, and proteome studies have found upregulation of glycolysis and downregulation of oxidative respiration and differently expressed proteins like SM-22α, Rab37, haptoglobin, and Rho-GDIα. Although the ‘omics’ studies in endometriotic lesions require invasive procedures and will not provide biomarkers directly translatable into the clinical practice, the knowledge obtained from these studies enables more complex insight into the possible mechanisms of endometriosis pathogenesis.

'Omics' Studies on Blood and Body Fluids and Novel Endometriosis Biomarkers

The ultimate goal of 'omics' studies in endometriosis is to find robust and specific non-invasive biomarkers with acceptable sensitivity and specificity and preferably from easily assessable sources like blood and body fluids. Endometriosis biomarkers have been sought from peripheral blood (whole blood, plasma, serum), menstrual blood, endometrial fluid, peritoneal fluid and urine samples, and more than 100 markers, among them annexin V, VEGF, CA-125 and sICAM-1/or glycodelin, glycoproteins, inflammatory and non-inflammatory cytokines, and angiogenic and growth factors have been reported but with inconsistent and contradictory results [4, 5, 85, 86].

The first large-scale genomic study from blood was published in 2010, and since then, numbers of SNP microarray-based genome wide association studies (GWAS) from genomic DNA, together with following replication studies, have been conducted to reveal associations between common SNPs and endometriosis [87–95]. Previous meta-analysis included more than 11,506 patients and 32,678 controls and found six loci (rs12700667 on 7p15.2, rs7521902 near *WNT4*, rs10859871 near *VEZT*, rs1537377 near *CDKN2B-AS1*, rs7739264 near *ID4*, and rs13394619 in *GREB1*) that had consistent effects across different populations [96]. In a recent study by Steinthorsdottir et al., 1840 women with endometriosis and 129,016 controls were included into GWAS, and in addition to the previously reported susceptibility loci [96], also two new loci on 4q12 (rs17773813) upstream of *KDR* encoding vascular endothelial growth factor receptor 2 (VEGFR2) and rs519664 in *TTC39B* gene on *9p22* were identified [95].

Although GWAS have provided valuable information about novel candidate genes and genome regions, the effect sizes for the associated variants are quite moderate (odds ratios between 1.0 and 1.2), and apparently these common variants do not provide any molecular markers for direct diagnostic or prognostic tests. However, it is very likely that in the case of a common diseases, such as endometriosis, the rare variants (minor allele frequency <0.05) could contribute to the risk of the disease [97].

In addition to GWAS, high-resolution SNP arrays provide the opportunity to assess inherited copy number variations (CNVs) that normally exist in all tissues and may potentially contribute to genetic predisposition of common diseases. Although many disease-related CNVs have been described, large population-based CNV studies have also found substantial variability in CNV distribution in healthy individuals [98–100], challenging the findings of CNVs responsible for the development of a complex diseases. Genomic CNVs in endometriosis have been investigated so far only in two large-scale studies [101, 102]. Chettier et al. conducted a case-control study of 2126 surgically confirmed endometriosis cases and 17,974 controls of European ancestry and found no significant differences in CNV counts, excess of large CNVs, and gene-based CNVs between controls and patients [101]. However, the locus-specific analysis revealed 22 rare CNVs that were detected in

6.9% of the affected women compared to 2.1% of the general population. Three out of 22 CNVs passed a genome-wide *P*-value threshold, namely, a deletion at *SGCZ* on 8p22, a deletion in *MALRD1* on 10p12.31, and a deletion at 11q14.1 [101]. Recently, six sub-telomeric putative novel CNV loci in regions 1p36.33, 16p13.3, 19p13.3, 20p13, 17q25.3, and 20q13.33 from pooled DNA samples of 100 patients and 50 controls were reported by Mafra et al. [102]. Though the genomic studies have not been very successful in uncovering the pathogenesis of endometriosis or finding disease-specific biomarkers, it is very likely that the availability of more advanced methodologies (exome sequencing and whole-genome sequencing) will provide more detailed information about the genomic background of endometriosis in the near future.

Transcriptome studies have focused on determining circulating miRNAs in blood serum or plasma. miRNAs are considered as good candidates for biomarkers because cell-free miRNAs are shown to be stable in different body fluids [103]. In healthy individuals, the miRNA profile in serum is similar to that of circulating blood cells, but in the case of physiological or pathological changes, the levels of miRNAs in serum may differ [103]. Thus, alterations in the miRNA levels may possibly be used as biomarkers for endometriosis [104]. Although more than 200 potential non-invasive miRNA biomarkers have been proposed for endometriosis [105–110], the results are still inconsistent between studies, and only 12 miRNAs (miR-548b-5p, miR-92a, miR-320d, miR-139-3p, miR-122, miR-145*, miR-15b, miR-21, miR-572, miR-9*, miR-199a-5p, miR-342-3p) have been found to be differentially expressed in at least two studies. Not a single miRNA alteration has been confirmed in all studies. As there are only six global miRNA studies published to date with moderate numbers of participants involved, thus the real diagnostic potential of miRNAs in endometriosis is not fully discovered, and further studies, using additionally more advanced sequencing techniques to fully describe the wide spectrum of miRNAs, are needed.

Proteomics analyses have been extensively conducted to identify endometriosis-specific biomarkers from blood plasma [111, 112], serum [37, 44, 113–124], urine [125–128], endometrial fluid aspirate [129], menstrual blood [130], and peritoneal fluid [128, 131–137]. Although the number of serum-based studies is quite remarkable, the correlation between the different studies is relatively small. Nevertheless, some of the studies have identified a signature of peptides/proteins that could discriminate patients from controls with relatively high sensitivity and specificity. Jing et al. found two proteins (5830 m/z and 8865 m/z) in serum samples that were significantly more abundant in patients than in healthy women, and the signature of these two proteins offered diagnostic potential with a sensitivity of 86.7% and specificity of 96.7% [115]. A following study by Zheng et al. found three peptide peaks (5988,7; 7185,3; 8929,8 m/z) in serum that distinguished endometriosis patients in test and training sets with a sensitivity of 89.3–91.4% and a specificity of 90–95% [113]. A study conducted by Long et al. reported 13 serum proteins with significantly different levels between controls and patients and proposed one promising peptide (4180 Da) with 100% specificity and 100% sensitivity [114]. In the most recent study by Dutta et al., two proteins, HP and A1BG, were found to be effective

for the diagnosis of stage II, III, and IV endometriosis, with a sensitivity of 68–92% and a specificity of 84–96% [124]. In addition to the large number of serum proteome studies, a few plasma studies have been published [111, 112]. Fassbender et al. found that a model based on five protein/peptide peaks (2058; 2456; 3883; 14,694 and 42,065 m/z) discriminated ultrasonography-negative endometriosis with a sensitivity of 88% and a specificity of 84% [111]. A study by Liu et al. found 20 protein peaks that were up- or downregulated in the plasma of endometriosis patients [112]. Overall, the blood-based proteomic studies have brought out several potential biomarkers with relatively high sensitivity and specificity. However, it should be pointed out that the methods most commonly used in biomarker identification, SELDI-TOF-MS and MALDI-TOF-MS, have several shortcomings like limited mass range, and these methods do not provide direct protein identities. Therefore, for further biomarker discovery, other methods that enable identification of proteins (such as tandem mass spectrometry) are needed [138].

From the clinical perspective, urine that is simple to collect would be the most preferable source for disease-specific biomarkers. Similarly to other fluids, urine contains peptides and proteins that may reflect disease status and can be easily measured by proteomic methods. Indeed, proteomic studies from urine [125–128] have provided promising results. The study by Tokushige et al. found that cytokeratin-19 levels were not influenced by the menstrual cycle phase or disease severity and were elevated in patients with endometriosis [126]. The following study by El-Kasti et al. identified six peptides influenced by disease severity and menstrual cycle phase when controls were compared with moderate-severe endometriosis patients, and seven peptides when patients with minimal-mild disease were compared to moderate-severe endometriosis patients [125]. The study by Cho et al. found 22 protein spots with differential expression in patients, among them is urinary vitamin D-binding protein (VDBP). However, the diagnostic potential of VDBP in endometriosis alone or combined with serum CA-125 remained moderate [127]. No specific urine proteomic biomarkers that could discriminate patients and controls were found in a study by Williams et al. [128].

The full potential of menstrual blood as a source of biomarkers is yet to be found. Although the collection of menstrual blood is fairly complicated, it could reflect the physiological and molecular environment of the pathologically altered endometrial cells of endometriosis patients more precisely compared to the peripheral blood (reviewed in [138]). However, there is only one study using menstrual blood for biomarkers research published so far [130]. This study identified three differentially expressed proteins as endometriosis-specific markers: collapsin response mediator protein 2, ubiquitin carboxyl-terminal hydrolase isozyme L1, and myosin regulatory light polypeptide 9 [130]. Additionally, the study reported higher expression of stem cell marker gene transcripts (*Oct-4*, *CXCR4*, *SOX2*, and *c-MET*) in the menstrual blood of patients with endometriosis [130]. The higher expression of stem cell markers in menstrual blood of women with endometriosis may indicate the importance of these markers in implantation process of endometriotic lesion.

Microenvironment of the peritoneal cavity is thought to be one important factor influencing the capability of endometrial cells to implant into the peritoneal cavity.

Further, there is strong evidence that dysregulation of peritoneal immunological and proinflammatory systems, and also alterations in angiogenesis processes may play a crucial role in the progression of the disease. Therefore, a number of proteome studies of peritoneal fluid have been conducted, and several potential biomarkers have been proposed [128, 131–136]. Silicano et al. studied peritoneal fluid in patients with different disease stages and found a pattern of peptides corresponding to fibrinogen alpha chain that were more frequently present in women with moderate-severe endometriosis [131]. Study by Wölfler et al. identified 11 differentially regulated proteins that might have an impact on the development and establishment of endometriotic lesions [136]. Ferrero et al. found nine proteins mostly involved in the immune response (e.g. serotransferrin, complement C3, serum amyloid P-component, alpha-1-antitrypsin, and clusterin) with significantly higher expression in infertile endometriosis patients than in infertile controls [135]. Williams et al. detected a number of proteins with metabolic functions, such as proteins involved in glucose metabolism (phosphoglycerate kinase-1, fructose-bisphosphate aldolase A, transaldolase, triosephosphate isomerase, malate dehydrogenase) and glutathione *S*-transferase P which is involved in detoxification [128]. Summing up, although studies in peritoneal fluid have provided some insight into the pathogenesis of endometriosis, the concordance between the results of different studies is non-existent, and to date, there are no specific reliable biomarkers for diagnostic purposes. Also, the diagnostic potential of peritoneal fluid biomarkers in clinical practice is debatable due to the invasive nature of the peritoneal fluid collection.

Beside other ‘omics’ technologies, metabolomics has great potential to become a new frontier in endometriosis biomarkers research, as global changes in measurable, low-molecular-weight products of metabolism are thought to be good indicators of health status. Although the concentration of circulating metabolites provides integrative information about the tissue function within the larger context of the organism, the global metabolic profile is influenced by a number of dependent variables, such as environmental factors, altered activities or levels of enzymes, genetic factors, and lifestyle factors including diet, drugs, exercise, microbiota, hormonal homeostasis, and age (see review [139]), thereby challenging the finding of disease-specific metabolites. To date, only a few global metabolome studies using either blood serum or plasma of patients and controls have been conducted [140–143]. These studies have proposed several potential metabolites with a good diagnostic potential for endometriosis. Vouk et al. proposed a model including hydroxyl sphingomyelin (SMOH C16:1) and phosphatidylcholine/ether-phospholipid ratio (PCaa C36:2/PCae C34:2) that discriminates the ovarian endometriosis patients from controls with a sensitivity of 90% and a specificity of 84% [142]. Dutta et al. found 13 metabolites that discriminated minimal-mild endometriosis patients from healthy women with a sensitivity of 82% and specificity of 91% [141]. The study by Jana et al. found 15 metabolites showing a sensitivity and specificity of 92.83% and 100%, respectively [140]. In the most recent study by Ghazi et al., several metabolites such as 2-methoxyestradiol, 2-methoxyestrone, dehydroepiandrostion, andro-

stenedione, and cholesterol showed significant increase in the endometriosis group compared to control group [143]. As all these four studies reported different metabolites that discriminate diseased and healthy women with high sensitivity and specificity, future studies including larger number of participants and different types of endometriosis are needed.

The research in the field of non-invasive biomarkers has been comprehensive and continuously ongoing, and valuable knowledge will be obtained piece by piece. The results from transcriptome, proteome, and metabolome studies are encouraging and hold a great promise for endometriosis biomarker discovery.

Perspectives in Single-Cell 'Omics'

The remarkable progress in 'omics' technologies using DNA or RNA from small amount of cells or even single cells has revolutionised our conceptual understanding of biological diversity of human cells and has allowed to take a closer look into the single-cell genome, proteome, epigenome, and proteome in health and disease [144].

So far all 'omics' studies in endometriosis have operated on the level of systemic or multicellular analysis, and results of these studies inevitably show any changes in a greatly diluted fashion, and therefore the potential of single cell technologies in endometriosis research is yet to be realised. The traditional approaches in endometriotic lesion research have not provided any clear consensus about the genetic, transcriptomic, and epigenetic changes inside the lesions, and therefore, the 'omics' information from single cells or from homogenous cell populations from lesions (e.g. endometrial epithelial and stromal cells, stem cells, endothelial cells, monocytes, NK cells, lymphocytes, and dendritic cells) could offer new prospects to reveal the true disease-specific molecular changes (Fig. 2).

The major challenge of using single-cell technologies is not related to the research methodology or data analysis per se but is rather associated with obtaining specific single cells or cell populations from lesion biopsy. There are already some good examples of using fresh tissue biopsies for single-cell RNA sequencing [145–147], and the methodology can be transferred from these studies to endometriosis research. Also, there is great potential to use combinations of fluorescently labelled antibodies (e.g. CD10, CD9, and CD13 are previously shown to be markers of endometrial stromal and epithelial cells [148]) and fluorescence-activated cell sorting (FACS) for isolating single cells or cell populations from lesions. Furthermore, cell populations could be isolated from lesions using LCM technique, but as DNA and RNA quality obtained by this methodology varies a lot [149, 150], this method would probably not be the first choice. In addition to the lesion and endometrial single-cell studies, specific cells originating from blood or body fluids (like monocytes, NK cells, lymphocytes, granulocytes, endothelial cells, and progenitor cells) could offer new interesting perspectives to uncover pathologic changes related to the disease.

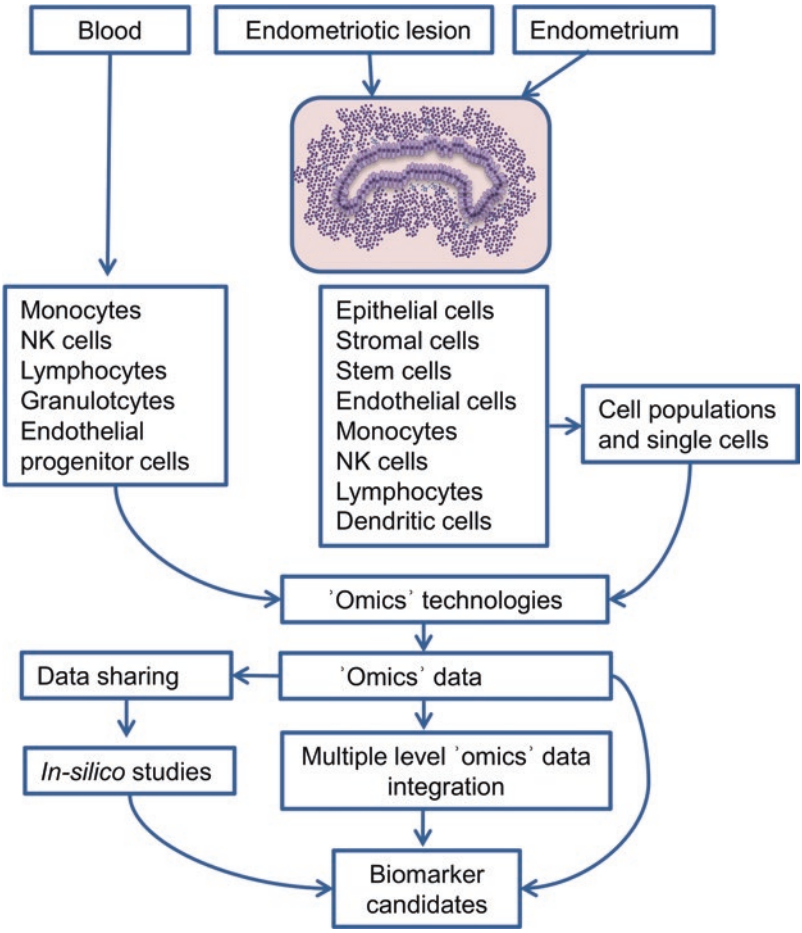

Fig. 2 Single-cell 'omics' in a search of endometriosis-specific biomarkers

Although the advantages of single-cell 'omics' research in endometriosis are not utilised yet, it could be assumed that traditional transcriptome and epigenome studies will progress from the multicell level to single-cell level in the nearest future.

Systems Biology and Integrative 'Omics' Studies in Endometriosis

The number of 'omics' studies in endometriosis is rapidly increasing, and the massive amount of 'omics' data is becoming an immense challenge to the researchers. However, no single 'omics' analysis can fully resolve the complexity of the biology

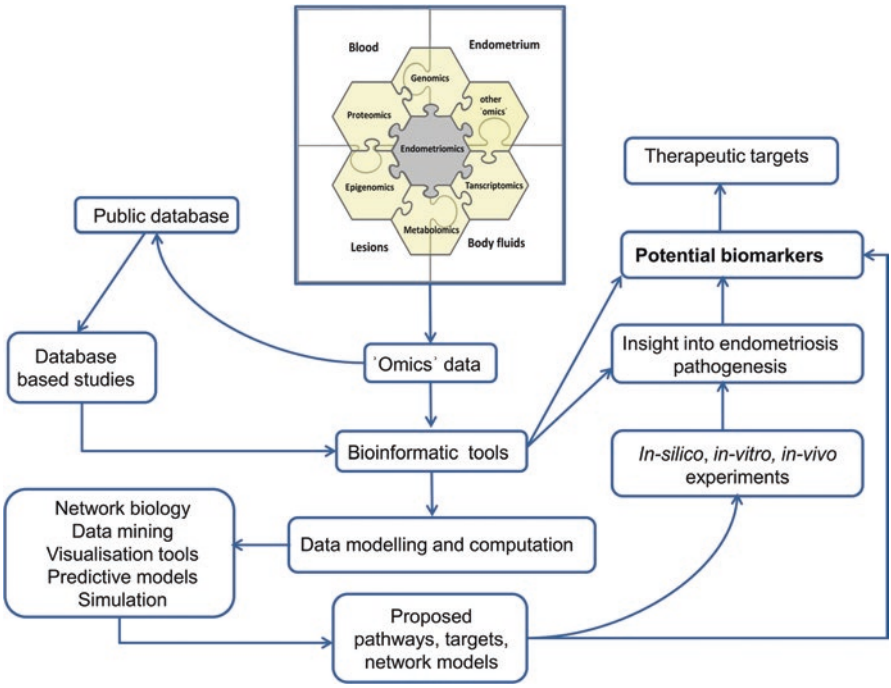

Fig. 3 Integrated systems biology approach for potential endometriosis biomarker discovery. The idea of systems biology and concept of integration of 'omics' data in a search for endometriosis specific biomarkers are illustrated

behind the disease development. Therefore, one of the main tasks for systems biology is the integration of large amounts of different types of data in order to understand the functional principles and dynamics of cellular systems and unravel the complexity of molecular networks in health and disease. Integration of multiple layers of 'omics' data requires network analysis and annotation of the involved pathways to capture meaningful information from genomics, epigenomics, transcriptomics, proteomics, metabolomics, and other 'omics' studies. After integration of single 'omics' data with computational engineering, modelling, and simulation and visualisation tools, new theoretical models can be created that provide comprehensive interpretation of integrated data (Fig. 3).

In endometriosis research, the first attempts to use systems biology and integrated data and knowledge from different 'omics' studies have been done [12, 13, 54–56, 67, 73, 151]. Most of these studies have combined epigenome data with gene expression data in order to reveal correlations between DNA methylation and gene expression. Borghese et al. found 35 genes that shared alterations both in methylation and gene expression levels [55]. The following study by Yamagata et al. observed a relationship between altered methylation and mRNA expression in 75

genes from cultured stromal cells from eutopic endometria and endometrioma, including steroidogenesis-related genes *NRA1*, *STAR*, *STAR6*, and *HSD17B2* [56]. The results of this integrative analysis suggested that aberrant DNA methylation of the key steroidogenesis-related genes causes aberrant gene expression leading to the development of endometriosis. The transcriptome and epigenome data interaction modelling of stromal cells originating from eutopic and ectopic endometria of patients together with stromal cells from healthy endometria was performed by Dyson et al. [54]. They identified hundreds of genes, correspondent to thousands of differentially methylated CpGs that were aberrantly methylated and differentially expressed, among them the *HOXA* cluster, *ESR2*, *NR5A1*, and *PGR* genes. Furthermore, the authors classified the list of these genes by protein function and found that the only proteins that reached a statistical significance were transcription factors, such as GATA transcription factors and transcriptional coregulators of the GATA family. They proposed that GATA2 could regulate genes important for the decidualization process, whereas GATA6 promotes an endometriotic phenotype via regulation of steroidogenesis in endometriotic cells [54]. Sun et al. combined transcriptomic data of eutopic and ectopic endometria from mRNA and lncRNA microarray and found hundreds of lncRNAs that were co-expressed with thousands of mRNAs [67]. Hawkins et al. performed the first transcriptome-miRNome analysis of endometriomas and eutopic endometrium in order to narrow down genes that were functionally targeted by miRNAs [73]. The combined analysis revealed several potential biologically important pathways involved in cellular development, connective tissue development and function, and cellular growth and proliferation including *TGFβ* and mitogen-activated kinase 1 [73]. Completely novel approach to uncover regulatory changes in endometriosis was applied by Yang et al. [151]. They used integrative analysis of gene expression data from two different datasets combined with data from transcription factor (TF) gene regulatory interactions database (identified from ChiP-seq or ChIP-chip experiments available from ChEA database) to find endometriosis-associated TFs. This data was further combined with data from protein-protein interaction database (Interologous Interaction Database (I2D) and data from Ravasi et al. [152]) to create integrated regulatory network for understanding molecular mechanisms involved in endometriosis [151]. Authors identified a network of known TFs such as androgen receptor and estrogen receptor α and β participating in endometriosis pathogenesis. Also, several new TFs, such as FOXA2 and TFAP2C, were identified and validated in mRNA and protein level [151].

In conclusion, the 'omics' studies in endometriosis have provided the researchers with a substantial amount of data, but clearly, the full potential of this data is not entirely utilised. Although the amount of 'omics' datasets in publicly available repositories (Gene Expression Omnibus – GEO, etc.) is considerable, researchers still prefer using their own small datasets [153]. While the 'omics' studies in endometriosis are relatively small in size, future studies should use the advantage of the publicly available pre-existing data to raise the power, credibility, and reliability of the findings and also to find new biomarkers without the wet-lab costs.

Study Design in 'Omics' Studies in Endometriosis

The outcomes of 'omics' studies have also highlighted the shortcomings related to study design that may be the reason for the poor overlap between the results from different studies.

Large variability in tissue collection, processing, and storage methodology, poor description of patient phenotype data, small size of study groups, and differences between 'omics' platforms, together with data analysis and interpretation differences, are likely to result in bias and measurement error between studies. Standardised sample collection is crucial in biomarker discovery, as small differences in the processing and handling of biological samples can lead to the pre-analytical bias and might have a huge effect on analytical reliability and reproducibility [154]. The importance of harmonising standard operating procedures for collecting phenotypical data, and for sample collection, processing and storage is discussed in detail in the publications of the World Endometriosis Research Foundation Endometriosis Phenome and Biobanking Harmonisation Project (WERF EPHect) [155–157]. The main goals of the harmonised data and sample collection are not only to facilitate large-scale international collaborations and decrease cross-centre variability but also to collect information for future studies addressing specific research questions on different patient subtypes. The minimum and standard recommendations for surgical phenotype data collection include information about menstrual cycle, hormone treatment, history of previous endometriosis surgery, as well as any imaging findings before the procedure and the type and duration of the procedure; the extent, exact location, and colour of endometriotic lesions; and video/photo documentation of surgery [157]. In addition, the WERF EPHect provides precise recommendations for collecting, processing, and storing fluid biospecimens (plasma, serum, saliva, urine, endometrial/peritoneal fluid, and menstrual effluent) and tissue specimens (ectopic and eutopic endometrium, peritoneum, and myometrium) and for collecting biospecimen data, including information about the menstrual phase on the day of the sample collection, menstrual cycle regularity, timing of the next menstrual cycle, administration of any premedication or anaesthetics, and also information about the weight, height, and waist and hip circumference [155, 156]. It is strongly advised to follow the WERF EPHect published guidelines for designing new studies, as the well-characterised phenotypic datasets could be used to answer various current and future research questions in endometriosis.

One of the concerns related to the design of 'omics' studies is the definition of the endometriotic lesion. Majority of the studies have compared biopsied lesion samples and endometria; however, endometriotic lesions often contain only a small proportion of endometrial glands and stromal cells and a large proportion of surrounding tissue. Histological evaluation of biopsies is routinely used in everyday practice, and several studies have demonstrated that approximately 30–50% of surgical specimens removed during laparoscopy are not confirmed by histological

assessment [158–161]. Even in the case of histologically confirmed biopsied endometriotic lesions, the proportion of endometrial tissue is variable. As different tissue types have their own molecular signature, the comparison between endometrial tissue and lesions that contain a mixed population of endometrial and peritoneal or ovarian tissue will not reveal disease-specific alterations but may rather reflect molecular signature of the surrounding tissue [76]. Therefore, future 'omics' studies of lesions should focus on pure cell populations or single-cell analyses instead of studying the entire endometriotic lesion to reveal the true molecular signature of endometriotic cells.

Selecting proper controls for biomarker discovery studies is one of the key issues that need to be resolved. Currently, endometriosis studies have applied different strategies for choosing controls and have included either fertile women undergoing laparoscopic sterilisation, women undergoing laparoscopy because of infertility, hospital-based controls (women with various indications for laparoscopic procedure), and also self-reported disease-free population-based controls [162]. Selecting controls for searching biomarkers from blood and body fluids is problematic, and there is never one ideal control group, as all above-mentioned options have their pros and cons. For example, laparoscopically confirmed endometriosis-free controls usually suffer from other diseases; a self-reported disease-free population-based control group could contain a number of undiagnosed cases and thereby dilute the disease risk factor effects.

The cost of the high-throughput technologies is relatively high, the sample sizes in 'omics' studies are rather small, and therefore the studies are underpowered to take into account the variance of individual measurements. Thus, it is strongly encouraged to maximise the sample sizes in 'omics' studies by collaborating with other workgroups and sharing either phenotypically well-described samples or data. In addition, there are other study type-specific standards for minimum information, including MIAME (Minimum Information about a Microarray Experiment), MIAPE (Minimum Information about a Proteomics Experiment), MIGS-MIMS (Minimum Information about a Genome/Metagenome Sequence), MIMIx (Minimum Information about a Molecular Interaction eXperiment), MINISEQE (Minimum Information about a high-throughput Nucleotide Sequencing Experiment), and CIMR (Core Information for Metabolomics Reporting). These guidelines must be followed to ensure the interpretability of the experimental results generated using 'omics' technologies [163, 164].

In conclusion, a good study design in endometriosis 'omics' studies includes setting an innovative study hypothesis, defining phenotypically well-described controls/cases, calculating study power that takes into account individual measurement variance, acceptable false-positive rate, and desired power of the used platform, collecting phenotypical data and biospecimens according to the guidelines of WERF EPHect, identifying risk factors and confounders, assessing sample quality and quantity, following the protocols for 'omics' technologies according to the specific guidelines (MIAME, MIAPE etc.), considering technical duplicates and statistical

methods, describing databases for data analysis, validating results using alternative technologies, presenting data (e.g. GEO database), and addressing limitations/strengths of the study.

Conclusions and Future Perspectives

The 'omics' revolution in endometriosis research is ongoing, and around 120 studies applying the high-throughput 'omics' technologies have been published to date. Significant advances in 'omics' technologies have been made to discover potential biomarkers for endometriosis; however, most of the results have not been replicated in other studies, and the practical value of the proposed biomarkers is still limited. Though the genomic studies in endometriosis have not been very successful for finding potential biomarkers, the transcriptomic studies of endometriosis have provided some clues about the potential disease related pathways. Also, the results from proteome studies have been encouraging and hold a great promise for non-invasive biomarker discovery. Furthermore, great perspectives for future endometriosis biomarkers discovery are related to metabolomics and epigenomics as these fields are still poorly covered and harbour immense opportunities. Also, the advantages of single-cell transcriptome and epigenome studies should be carefully considered when planning future research.

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