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An array of possibilities: Multiplex immunoassays in endometriosis

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Abstract

Endometriosis is a complex disorder which is diagnosed through laparoscopy. The invasive nature of this approach has urged the field to explore the possibility of developing a biomarker-based diagnostic test in blood. Despite the realization that a panel of biomarkers may be required as the optimal diagnostic tool, the use of multiplex immunoassays has been limited in endometriosis. Multiplex assays range from small-scaled multiplex sandwich ELISAs in a planar or bead-based format to the more expanded antibody arrays employing direct sample labeling. The plethora of data generated from these arrays should preferably be examined using multivariate statistical analysis. With the constant development of multiplex technologies, future studies should focus on implementing these techniques, and combining them with multivariate statistical analysis in order to finally develop a non-invasive diagnostic test for endometriosis.

Key words

■ Endometriosis ■ Multiplex immunoassay ■ Planar analytical sandwich ELISA ■ Bead-based immunoassay ■ Label-based antibody array ■ Biomarker ■ Multivariate statistics

Introduction

Endometriosis is thought to be a complex disease, with a polygenic and environmental origin [1]. The current golden standard for the diagnosis of endometriosis is laparoscopy [2]. The drawbacks of this procedure have stimulated the search for a non-invasive biomarker-based test for endometriosis [3]. The diagnosis of a multifactorial disease is unlikely to be captured by a single biomarker [4]. Combining multiple biomarkers into a panel is more likely to have an increased accuracy [5], and thereby a greater diagnostic value [6]. Biomarkers and biomarker panels have been proposed for endometriosis [4, 7-9], but no panel has been validated for clinical application in peripheral blood [4], nor in endometrium [10].

Multiplex analysis is a useful tool to construct panels of biomarkers, because it allows the parallel measurement of a number of proteins in a low volume [11], thereby not wasting precious samples and allowing large sample sets [6]. Many efforts have been done to identify biomarkers with multiplex immunoassay techniques. The advantage of mutiplex immunoassay techniques over proteomic mass spectrometry methods is that they allow direct identification of the biomarkers, and therefore aid the transition to the validation phase [12]. Furthermore, the immunoassay techniques have higher detection sensitivities, lower cost and are more user-friendly [12, 13]. In this chapter, we describe three types of multiplex panels, summarized in Table 1 and Figure 1, namely the planar analytical multiplex sandwich Enzyme-Linked ImmunoSorbent Assay (ELISA), the multiplex bead-based immunoassay, and the label-based antibody array.

1. Multiplex immunoassay techniques and their use in endometriosis

1.1. Planar analytical multiplex sandwich ELISA

The planar analytical multiplex sandwich ELISA has been developed from the traditional ELISA to realize the simultaneous detection of a number of analytes [14]. The working

mechanism is similar to a classical ELISA and is set on the surface of a 96-well plate or on a glass or membrane coated slide. Chemiluminescence and in some cases fluorescent detection methods are employed because of the increased sensitivity compared to the traditional chromogenic detection [14, 15]. A sandwich approach is based on the interaction between a capture antibody for identification purposes, its corresponding antigen, and a detection antibody recognizing different epitopes of the same protein (Figure 1a) [16]. In endometriosis, a mini-array sandwich ELISA measuring 9 chemokines showed higher concentrations of macrophage inflammatory protein (MIP)-3β in peritoneal fluid (PF) of patients with endometriosis compared with controls suffering of primary infertility [17] (Table 2). Vodolazkaia *et al.* performed the multiplex chemiluminescent detection of osteopontin, insulin-like growth factor-binding protein (IGFBP)-3, and leptin in plasma [8]. Despite leptin and IGFBP-3 showing univariate statistical differences between cases and controls, they were not included in the panels ultimately selected after multivariate analysis to detect ultrasound (US)-negative endometriosis, and consisting of cancer antigen (CA)-125, vascular endothelial growth factor (VEGF), Annexin V, and glycodelin or soluble intercellular adhesion molecule (sICAM)-1 [8]. Beside the possibility to screen for potentially important markers for endometriosis, customized panels can be developed, validated, and subsequently implemented in diagnostics [18].One study compared the performance of a multiplex sandwich ELISA with routine clinical testing and singleplex ELISAs utilizing identical antibodies [19]. The multiplex ELISA correlated for four of eight proteins with routine testing, while it was reliable for five of eight proteins when compared to the single ELISAs [19]. Therefore, some of the variability between singleplex and multiplex can be attributed to the use of different antibodies [19]. Due to the sandwich design, specificity and sensitivity are usually good, however this format limits the number of detectable analytes to approximately 100 because of the cross-reactivity between antibodies [12].

1.2. Multiplex bead-based immunoassay

The principle of a multiplex bead-based immunoassay (Figure 1b) is based on the flow cytometric detection of a mix of fluorescently coded beads in suspension, coated with specific capture antibodies [14, 20]. The analyte is identified by the fluorescence of the bead, while it is quantified by the generation of a second fluorescent signal from the detection antibody [21]. Multiplex bead-based assays have been implemented in different fields of research [22], including endometriosis [8, 23-28]. The technique has been used extensively to detect the link between inflammation and endometriosis locally in PF samples [23-27] and to a lesser extent in serum [26] or plasma [8, 28] (Table 3). While Podgaec *et al.* could not detect any differences in the serum concentration of the cytokines, PF levels of interferon (IFN)-γ and interleukin (IL)-10 were found to be significantly upregulated in endometriosis after univariate analysis [26]. Another group also showed the significantly elevated expression of IL-10 in endometriosis patients versus controls , alongside IL-6 [27]. IL-6 was particularly elevated in patients with a more advanced disease stage. ROC curve analysis showed that only IL-6 had good discriminatory power showing an area under the curve of 0.853 for the subgroup of patients with stage III-IV endometriosis [27]. In correspondence with the previous study, Bersinger *et al.* reported the significant upregulation of IL-6 in stage III-IV endometriosis [23]. Additionally, IL-18 was significantly elevated in stage III-IV endometriosis, while eotaxin and monocyte chemoattractant protein (MCP)-1 were significantly elevated in both the stage III-IV subgroup and the group of all endometriosis patients (stage I-IV) [23]. Interferon gamma-induced protein 10 (IP-10) was significantly elevated in the I-IV group, but not in the III-IV subgroup [23]. No real multivariate statistics was applied, but multiplication of IL-18 and MCP-1 concentrations was reported to increase the power to discriminate endometriosis cases and controls [23]. In a study by Mier-Cabrera *et al.*, IL-1β, tumor necrosis factor (TNF)-α, IL-6, IL-8, IL-10, eotaxin, VEGF, MCP-1, and

regulated on activation, normal T cell expressed and secreted (RANTES) were all significantly increased in PF of women with endometriosis, while IFN-γ, interleukin 1 receptor antagonist (IL-1ra), and IL-15 were significantly decreased [24]. An unusual approach was used by Beste *et al.*: instead of performing disease stratification, as is usually done in biomarker research, unsupervised multivariate analysis using non-negative matrix factorization (NMF) was applied to develop a pattern of biomarkers (or a consensus signature) in PF [25]. After model building, its ability was checked to distinguish endometriosis patients from controls [25]. The consensus signature consisted of 13 elevated cytokines: IL-8, RANTES, macrophage migration inhibitory factor (MIF), IL-6, MCP-1, granulocyte-colony stimulating factor (G-CSF), monokine induced by gamma interferon (MIG), hepatocyte growth factor (HGF), IL-10, IL-16, IL-1ra, growth-regulated alpha protein (GROα), and IL-1β. 28 of 41 endometriosis samples did not show this consensus signature and could not be separated from the controls. The other 13 patients showed four or more elevated markers from the consensus signature [25]. This discrepancy within the endometriosis group may be due to the disease heterogeneity and the dynamic changes of lesion progression. While this consensus signature may not be optimal for a diagnosis of endometriosis, since it does not separate the patients and controls optimally, it may aid the clarification of the pathogenesis, as macrophages were shown to be at the core of the production of consensus cytokines [25]. Furthermore the signature may be helpful to identify women who might benefit from post-operative immune treatment [25].

Since a biomarker panel in peripheral blood is most likely to be clinically useful for the diagnosis of endometriosis, two articles on biomarkers in serum and plasma focused on the combination of multiplex immunoassays and multivariate analysis as important tools for model building [8, 28]. Othman *et al.* found no improved diagnostic performance of a panel consisting of IL-6, MCP-1, and IFN-γ, compared with IL-6 alone [28]. In contrast,

multivariate analysis was very instructive in a study published by Vodolazkaia *et al.* [8]. This study used, alongside the traditional single ELISAs, two types of multiplex panels: one based on sandwich ELISA (as mentioned in the paragraph 1.1.) and the second one based on the bead-technology. The samples were divided into a training set (model building) and a test set (model validation). When multivariate analysis was applied on a subset of endometriosis patients with endometriosis not detectable on pre-operative ultrasound (US negative endometriosis), the best models consisted of CA-125, VEGF, Annexin V, and glycodelin or sICAM-1 [8]. Of these markers, only VEGF was part of the multiplex assay. The other markers had been identified using traditional ELISAs (Annexin V, glycodelin, and sICAM-1) and a clinical laboratory test (for CA-125). The diagnostic performance of the panels was better than that of any single biomarker, further emphasizing the importance of comparing multiple markers with a multivariate statistical analysis [8].

The advantage of the bead-based method is the high level of multiplexing and the flexibility in array composition [20]. The result is generated from the read-out of multiple beads, rendering each bead as a duplicate, which is favorable for the accuracy and robustness of the assay [29]. Furthermore, the advantage of a solution-based bead system is the increased efficiency to capture antigens [22] and the broader dynamic range [14, 22]. Tarnok *et al.* compared the performance of a multiplex bead-based system with single ELISAs used in routine diagnostics and found that the results correlated highly, indicating a good reliability of the multiplex assay [22]. However, the assay might not perform optimally for all proteins and setting a common dilution factor may be problematic [18]. Different results of the same cytokines in separate studies may be due to varying robustness among the multiplex bead-based immunoassays [27]. This type of assay has been used in rheumatoid arthritis by Khan *et al.* who compared the performance of four different bead-based immunoassay kits [30]. Some different results were detected between the kits, but the authors contributed this mainly to the

differences in antibody pairs, antigens for generating the standard curves, and composition of sample diluents and assay buffers [30]. In most studies, general trends correlated well between bead-based assays and ELISAs [31]. However, variation on a quantitative level can be seen, except when identical antibodies are used [31]. Bersinger *et al.* stated that the assay was not validated for PF samples [23]. Markers in PF may be more suited to investigate pathogenesis rather than diagnostics, since PF is not readily accessible except during surgery.

When comparing the two most frequently used systems reported in this chapter, Bio-Plex (Bio-Rad) can measure up to 500 analytes in one run, while the maximum number of analytes measured for the Cytometric Bead Array (CBA, BD Biosciences) is reported as 30. This is due to the fact that CBA uses different intensities of one fluorophore to distinguish the beads, while Bio-Plex applies a ratio of two fluorophores, creating possibilities for a higher number of assays [31]. The advantage of the CBA system is that the results can be measured on a standard flow cytometer, while the Bio-Plex assay requires a Luminex instrument built on xMAP technology [31]. The advantage of the Bio-Plex system is that they can provide the entire system of assay kits, software, calibration, and validation tools, leading to a high degree of accuracy [28].

1.3. Label-based antibody array technology

The label-based antibody array is related to the DNA array technology for gene expression profiling. Essentially, it is a version of the planar arrays as discussed in paragraph 1.1, however the label-based antibody array technology is suited for a proteomic approach [11], which is defined by the systematic analysis of proteins in biological samples [32]. It is particularly useful to generate hypotheses. In essence, high density biomarker screening has been accomplished by up-scaling to a high density array. It is a high-throughput method, with rapid parallel detection of proteins and low sample requirement [33]. However, the sample throughput is usually lower than with the previously discussed methods (paragraphs 1.1 and

1.2), due to a generally higher cost of these assays [33]. An antibody array may be sandwich based [33] as in paragraph 1.1, but cross-reactivity between antibodies becomes a problem proportional to the size of the multiplex [16]. Direct labeling of sample eliminates this problem of cross-reactivity between antibodies because no detection antibody is needed, requiring only a capture antibody pre-printed onto the array surface [16] (Figure 1c). As there is no need for an antibody pair, more targets can be included [6]. Samples can be directly labeled with fluorescent dyes such as Cyanine (Cy)3 and Cy5, or labeled with biotin for later detection with a streptavidin-conjugated dye [16]. Alternatively, chemiluminescent detection is possible [34]. The advantage of the biotin label is that small molecules are less disruptive to the labeled protein than fluorophore tags [6], the possibility for signal amplification and the efficient labeling process, thereby contributing to an increased detection sensitivity [16]. Disadvantages of direct labeling include the possible masking of the epitope and the difficulty to obtain homogenous labeling among high abundant and low abundant proteins [6, 35]. Furthermore, specificity is lower in a single-antibody detection format than in a sandwich format, as cross-reactivity of antibodies to other antigens becomes a bigger issue [12, 16]. Detection sensitivity depends from array to array [16].

For cancer research the use of antibody microarrays has been described in serum, and in isolated proteins from tumor fragments and cultured cells [6]. To date, in endometriosis the use of large scale label-based antibody arrays has been limited to one study published by Hou *et al* [36] (Table 4). Cytokine array analysis was performed on pooled PF samples of controls, stage I-II endometriosis patients, and stage III-IV endometriosis patients. Sample size was limited, with the inclusion of 3 controls, 3 patients with stage I-II endometriosis, and 3 patients with stage III-IV endometriosis. All women had surgically confirmed absence or presence of endometriosis, were in the proliferative phase of the cycle, had no additional inflammatory or autoimmune diseases, and did not receive any hormonal medication prior to

sample collection [36]. 74 cytokines were 3-fold higher in the endometriosis pool versus the control pool. 4 cytokines were 3-fold lower in the endometriosis pool when compared with the control pool. When looking at the I-II pool versus the control pool, 96 cytokines had a 3-fold differential expression, including 91 increases and 5 decreases. Finally when stage I-II was compared with III-IV, 14 cytokines were increased 3-fold and 69 cytokines were decreased 3-fold in the III-IV group. These cytokines were hypothesized to be associated with the progression of endometriosis [36].

Advantages of the label-based antibody array approach are the fast identification of the proteins and the possibility for a quick validation using ELISA [16]. Disadvantages of this method include the reduced specificity and the assembly of multiprotein complexes, which may generate a strong disproportionate signal [11]. The complexity of human biological samples prevents the optimal detection of all proteins due to the large concentration range (pg/ml-μg/ml) [12]. Furthermore, assays results are usually semi-quantitative and need to be validated with ELISA.

1.4. Conclusion

Due to the increased awareness that multiple markers may be necessary to diagnose a complex disease such as endometriosis, the simultaneous measurement of different proteins in one sample is on its way to becoming an important tool in endometriosis research. Multiplex methods are more attractive than the classical single ELISAs, due to the cost-effectiveness and less need of precious biological samples [14]. They are more user-friendly, quicker, and more sensitive than mass spectrometry methods [13].

Disadvantages of multiplex methods are that they are less robust in plasma and serum samples, which are the interesting samples for a non-invasive diagnosis of endometriosis [14]. In biological fluids, a 'matrix effect' exists due to the presence of autoantibodies or due to

complicating factors affecting epitope binding, such as denaturation of epitopes by fluid components, glycosylation, soluble receptor binding and sample conditions [37]. Cross-reactivity may exist between capture antibody and antigens other than the one they are intended for [14], or between capture antibody and detection antibody [16, 31]. Additionally, background signals generated from non-specific binding of sample protein to the array may interfere with the signal measurements [12]. Furthermore, all analytes are measured in the same dilution and sample diluent, hence not creating an optimal environment for every detected analyte [29]. In the future, multiplex assays should be systematically compared with single ELISAs, and validated with single ELISAs [14]. Importantly, the complexity of the data warrants careful attention to study design and data analysis [14]. In the following paragraph, we will address the use and value of multivariate statistical analysis in endometriosis.

2. Multivariate statistical analysis: logistic regression (LR), least square support vector machines (LS-SVM), and Classification and Regression Tree (CART)

In biomedical science, studies are often designed to investigate the relationship between a response variable (e.g. disease status = 0 or 1) and one or more measured parameters (i.e. explanatory variables like biomarkers). Unlike univariate statistical methods which investigate each variable separately and link it to the response variable Y, multivariate methods allow to investigate the relationship of multiple variables simultaneously. Models based on multivariate statistical methods have a better performance for analyzing data and for making predictions.

In cases where the response variable is categorical (binary classification) logistic regression (LR) is the standard technique used for clinical classification problems [38, 39], as well as least square support vector machines (LS-SVM) [38, 40].

LR is a statistical method used in cases where we wish to model the probability of occurrence of an event such as the presence (Y=1) or absence (Y=0) of given disease (or disease status) like endometriosis [41]. This statistical method can be seen as an ordinary regression method, since it models the relationship between a response variable Y (presence or absence of endometriosis) and one or more explanatory variables (e.g. occurrence of particular plasma biomarkers) [41]. In ordinary regression, least squares are used for finding the best fit [42, 43]. However, in LR the response variable Y is categorical (e.g. disease status = 0 or 1) and therefore the underlying principle is quite different to that of ordinary regression [41]. LR estimates the probability p of the occurrence of an event, for instance the disease status of endometriosis, in terms of explanatory variables like for instance the concentration of plasma biomarkers. Because this relation is not presumed to be a linear function, the measure of association between the response and the explanatory variable is represented by an odds ratio (OR) instead of a multiplicative factor. Although LR intends to build a classification model that fits the data optimally, it may result in a model that overfits the data. Such models will fail to replicate future data. In other words, the model fits the training data very well but has a poor prediction accuracy when using an independent testing set. This can be explained by the presence of outliers and/or a small sample size influencing the model fitting [44], and consequently inducing a substantial number of misclassifications. Stepwise logistic regression is a semi-automated process for model building by successively adding or removing explanatory variables (for example particular plasma biomarkers) based solely on their individual statistic relevance (the statistic of their estimated coefficients).

Least square support vector machines (LS-SVM) are a class of machine learning methods related to standard support vector machines (SVM) [38, 40]. Both of them are class of kernel methods. They are based on the principal of statistical learning to solve classification problems. They are designed to generate both linear decision and more complex decision

boundaries. The commonly used complex decision (kernel function) is the radial basis function (RBF). This kernel requires optimizing the kernel parameter sigma (σ) and the regularization parameter γ, and this can be done using a 'gridsearch' approach. The kernel parameter sigma (σ) controls the curvature of the boundary and describes the nonlinearity of the data better than using a linear kernel. Figure 2 displays a case in which using a RBF kernel would be more appropriate than using a linear kernel, and therefore the nonlinearity in the data could be better described. However, this requires carefully tuning the parameter sigma (σ) and the regularization parameter γ to avoid overfitting [45].

Although both methods are extensively used in biomarker discovery, none of them is clearly superior when compared to the other. The ability of the nonlinear models to capture complex patterns, comes often at the cost of the interpretability. In contrast to LS-SVM, LR allows to build a model meaningful for medical diagnosis. LR allows to generate a simpler model by retaining only statistically relevant features (e.g. only a few plasma biomarkers). Although, theoretically having more features (e.g. more plasma biomarkers) should result in more discriminating power, practical experience has shown that this is not always the case [46]. The sensitivity of LR to outliers can lead to a model with poor prediction accuracy known as overfitting as discussed previously. To overcome this issue, Vodolazkaia *et al.* [8] proposed to apply a multivariate feature selection as a first step to reduce the effect of the irrelevant features. This was done in a bootstrapping approach, which uses sampling with replacement, where the data were randomly divided into a training set and in a test set [47]. In each loop, the training set was used to select relevant features by means of stepwise logistic regression. The loop was repeated 500 times. This resulted in selection of a panel of plasma biomarkers with improved sensitivity and specificity for the diagnosis of endometriosis as compared to the performance of any single plasma biomarker.

Seeber *et al.* [7, 48] used Classification and Regression Tree (CART) to classify endometriosis. CART analysis, which is a nonparametric statistical method, analyses all possible splits on each explanatory variables and selects the variable and cutoff value that identify the best splits which best classifies the subjects with regard to the endometriosis status. The tree can be "learned" by analysing the splits for each explanatory variables, and the split that maximizes the homogeneity of the cluster with respect to the endometriosis status is selected. Seeber *et al.* [48] proposed to use a reduced number of protein peaks by selecting only those with a given specificity and sensitivity, then using them to construct the classification tree. CART creates from each parent note 2 child notes and continues growing the tree, measuring the ability of the explanatory variables to classify subjects.

3. Future perspectives

In conclusion, we can state that combining multiplex arrays with multivariate statistical analysis holds great promise in our search for a non-invasive diagnosis of endometriosis. Multiplex techniques keep developing, allowing more sophisticated study designs. Until now, most studies have focused on the use of these multiplex immunoassays in PF. However, a diagnostic test in blood is more clinically useful. Therefore, serum and plasma samples should be investigated in a multiplex manner. Secondly, the sandwich-based techniques have been the focus of many of the studies reported here. For biomarker screening, the use of the label-based antibody array approach may be more advantageous due to its ability to screen a broad and high amount of analytes. As a next step, the biomarkers found with multiplex immunoassays should be validated.

Figure legend

Figure 1. Multiplex immunoassay methods.

a. Planar analytical multiplex sandwich ELISA. Capture antibodies against different antigens are immobilized on a 96-well plate or an array slide. The antigen is detected with an enzyme-linked detection antibody which converts a substrate to a product. This chemical reaction causes the emission of light for chemiluminescent detection. **b. Multiplex bead-based immunoassay.** Capture antibodies against different antigens are coupled to beads, each type with its own unique fluorescence. The method is solution-based and runs under sandwich ELISA conditions. The antigen is detected with a fluorescently labeled secondary antibody. The signal of the bead identifies the antigen, the signal of the secondary antibody determines the quantity. **c. Label-based antibody array technology.** Capture antibodies against different antigens are immobilized on an array slide. Samples are labeled, and those labels can be detected by a fluorescently conjugated molecule, eliminating the need for a detection antibody.

Figure 2. Complex versus simple linear decision boundary. Using LS-SVM machines with a complex decision boundary (e.g. using RBF kernel) is more appropriate in comparison to using LS-SVMs with a simple linear decision boundary (using a linear kernel) in cases of non-linear structures in the data.

Table 1. Overview of the different multiplex formats

	Multiplex Sandwich ELISA	**Multiplex bead-based assay**	**Label-based antibody array**
Design principle	Standard sandwich ELISA combined with microspot technology	Sandwich flow-cytometric bead-based immunoassay	Direct sample labeling combined with microspot technology
Sample preparation (apart from dilution)	No	No	Yes
Required antibodies	Antibody pair (sandwich format)	Antibody pair (sandwich format)	Capture antibody only
Format	96-well plate or microarray	96-well plate	Microarray
Method of detection	Chemiluminescence, fluorescence	Fluorescence (flow cytometry)	Fluorescence (laser scanner)
Application possible in clinical diagnostics	Yes	Yes	No
Use in endometriosis	[8, 17]	[8, 23-28]	[36]

Table 2. Overview of endometriosis studies using the multiplex sandwich-ELISA. Endo = endometriosis group. Ctrl = control group. * = subset of ultrasound-negative endometriosis patients

Reference	Sample type	Type of assay (company)	# analytes	Sample size	Multivariate statistics	Findings
Laudanski *et al.*, 2006 [17]	PF	Array spotted in 3 x 3 pattern using the Biochip Arrayer I^{TM} (Packard Instruments)[49]	9	Endo n = 24 Ctrl n = 18	No	↑ MIP-3β
Vodolazkaia *et al.*, 2012 [8]	plasma	Multiplexing sandwich ELISA system (Aushon Biosystems Search Light Assay Services)	3	Training set: Endo* n = 117 Ctrl n = 81 Test set: Endo* n = 58 Ctrl n = 40	Yes (LR, LS-SVM)	Proteins from this assay not in final panel for US negative endometriosis

Table 3. Overview of endometriosis studies using the bead-based immunoassay. Endo = endometriosis group. Ctrl = control group. * = subset of ultrasound-negative endometriosis patients

Reference	Sample type	Type of assay (company)	# analytes	Sample size	Multivariate statistics	Findings
Podgaec *et al.*, 2007 [26]	PF, serum	BD Cytometric Bead Array (Pharmigen, Becton Dickinson, Co.)	5	Endo $n = 65$ Ctrl $n = 33$	No	Only in PF: ↑ IFN-γ, IL-10
Wickiewicz *et al.*, 2013 [27]	PF	Cytometric Bead Array Th1/Th2 Human Cytokine Kit II (BD Biosciences)	6	Endo $n = 36$ Ctrl $n = 42$	No	↑ IL-10, IL-6
Bersinger *et al.*, 2012 [23]	PF	Bio-Plex platform (Bio-Rad Laboratories), selection from Human Cytokine panels I and II	13 (group I) + 5 (group II)	Endo $n = 101$ Ctrl $n = 32$	No	↑ IL-6, IL-18, eotaxin, MCP-1 Luteal phase: ↑ IL-12(p70), ICAM-1, GRO-α; ↓ eotaxin
Mier-Cabrera *et al.*, 2011 [24]	PF	Bio-plex human cytokine assay (Bio-Rad)	16	Endo $n = 32$ Ctrl $n = 30$	No	↑ IL-1β, TNF-α, IL-6, IL-8, IL-10, eotaxin, VEGF, MCP-1, RANTES; ↓ IFN-γ, IL-1ra, IL-15
Beste *et al.*, 2014 [25]	PF	Bio-plex Group I 27-plex, Group II 21-plex (Bio-Rad Laboratories)	27 (group I) + 21 (group II)	Endo $n = 57$ - No med $n = 41$	Yes (NMF)	Consensus signature: IL-8, RANTES, MIF, IL-6, MCP-1, G-CSF, MIG, HGF, IL-10, IL-16, IL-

				- Progestins n = 16 Ctrl n = 20		1ra, GROα, IL-1β
Othman Eel et al., 2008 [28]	Serum	Bio-Plex Protein Array System (Bio-Rad)	6	Endo n = 68 Ctrl n = 70	Yes (regres-sion analysis	↑ MCP-1, IL-6, IFN-γ
Vodolazkaia *et al.*, 2012 [8]	Serum	Bio-Plex Protein Array System (Bio-Rad)	17	Training set: Endo* n = 117 Ctrl n = 81 Test set: Endo* n = 58 Ctrl n = 40	Yes (LR, LS-SVM)	Only VEGF included in final marker panel for US negative endometriosis

Table 4. Overview of endometriosis studies using the label-based antibody array. Endo = endometriosis group. Ctrl = control group

Reference	Sample type	Type of assay (company)	# analytes	Sample size	Multivariate statistics	Findings
Hou *et al.*, 2009 [36]	PF	Biotin Label-based Human Antibody Array I (RayBiotech)	507	Endo n = 6 Ctrl n = 3	No	↑ 74 ↓ 4

Abbreviations

CA-125 = cancer antigen-125

CBA = Cytometric Bead Array

Ctrl = control

Cy = Cyanine

ELISA = Enzyme-Linked ImmunoSorbent Assay

Endo = endometriosis

G-CSF = granulocyte-colony stimulating factor

GROα = growth-regulated alpha protein

HGF = hepatocyte growth factor

IFN = interferon

IGFBP-3 = insulin-like growth factor-binding protein-3

IL = interleukin

IL-1ra = interleukin 1 receptor antagonist

LR = logistic regression

LS-SVM = least square support vector

MCP-1 = monocyte chemoattractant protein-1

MIF = macrophage migration inhibitory factor

MIG = monokine induced by gamma interferon

MIP-3β = macrophage inflammatory protein-3β

NMF = non-negative matrix factorization

OR = odds ratio

PF = peritoneal fluid

RANTES = regulated on activation, normal T cell expressed and secreted

RBF = radial basis function

sICAM-1 = soluble intercellular adhesion molecule-1

SVM = support vector machines

TNF-α = tumor necrosis factor-α,

US-negative endometriosis = ultrasound-negative endometriosis

VEGF = vascular endothelial growth factor

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