Multiplex immunoassays in endometriosis: An array of possibilities

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1. ABSTRACT

Multiplex immunoassays 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 could be of great interest to understand a complex disorder such as endometriosis. Multiplex immunoassay analysis may provide information on disease pathology and may lead to improved, timely diagnosis. Until now, the use of multiplex immunoassays has been limited in endometriosis. With the constant development of multiplex technologies, future studies should focus on implementing these techniques, and combining them with multivariate statistical analysis. In this review, we provide an overview of multiplex immunoassay methods used in endometriosis studies and the data sets acquired by these methodologies. These data and future studies might provide novel insights for biomarker discovery and investigation of the pathogenesis in endometriosis.

2. INTRODUCTION

In this review, we highlight several multiplex immunoassay technologies and their application in endometriosis research. Endometriosis is thought to be a complex disease, with a polygenic and environmental origin (1). Multiplex analysis, allowing measurement of multiple markers simultaneously, may give better insights into the biology of the disease and allow accurate diagnosis. Sampson's theory of retrograde menstruation is the most commonly cited hypothesis for the pathogenesis of endometriosis (2). However, retrograde menstruation is a common phenomenon, occurring in up to 90% of women of reproductive age, whereas endometriosis has a prevalence of 10% in women of the same age category (3). Therefore other mechanisms are believed to be involved in disease genesis and progression (3). These mechanisms may be elucidated by identification of protein signatures in bodily fluids of women with endometriosis. The current golden standard for the diagnosis of endometriosis is laparoscopy (4). The drawbacks of this procedure, such as the risk associated with surgery and its contribution to the diagnostic delay of 8-11 years, have stimulated the search for a non-invasive biomarker-based diagnostic test (5). The diagnosis of a multifactorial disease is unlikely to be captured by a single biomarker (6). Indeed, the clinical utility of individual biomarkers for endometriosis has been limited. CA-125 has shown promising results, but is also an ovarian cancer marker (7). Combining multiple biomarkers into a panel is more likely to have an increased accuracy (8), and thereby a greater diagnostic value (9). Biomarkers and biomarker panels have been proposed for endometriosis (6, 10-12), but no panel has been validated for clinical application in peripheral blood (6), nor in endometrium (13). Ideally, in routine clinical practice, a

diagnostic test is highly reproducible, rapid, and easily performed. Measuring biomarkers in peripheral blood, or even peritoneal fluid (PF) as semi-invasive diagnostic method, has the opportunity to greatly advance and simplify diagnosis (5, 7). In other complex diseases, the benefit of multiplex immunoassays has been proven. For example, in rheumatoid arthritis, multiplex immunoassays have identified about 20 differentially expressed cytokines and related mediators (14). A commercially available multiplex panel of 12 protein biomarkers has been developed to monitor disease activity in rheumatoid arthritis (14, 15).

Multiplex analysis allows the parallel measurement of a number of proteins in a low volume (16), thereby not wasting precious samples and allowing large sample sets (9). Therefore, multiplex analysis is more rapid and cost-effective than conventional singleplex Enzyme-Linked ImmunoSorbent Assays (ELISAs) (17). The advantage of multiplex immunoassay techniques over proteomic mass spectrometry based methods is that they allow direct identification of the biomarkers without further need of dedicated sample pretreatment, and therefore aid the transition to the validation phase (18). Furthermore, the immunoassay techniques have higher detection sensitivities, lower cost and are more user-friendly (18, 19). A general disadvantage of multiplex methods is the lack of robustness in plasma and serum samples (17). 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, soluble receptor binding and sample conditions (20, 21). Cross-reactivity may exist between capture antibody and antigens other than their target (17), or between capture antibody and detection antibody (22, 23). Additionally, background signals generated from non-specific binding of sample protein to the array may interfere with the signal measurements (18). Furthermore, all analytes are measured in the same dilution and in the same sample diluent, thereby not providing an optimal environment for every detected analyte (24).

In this review paper, we describe three types of multiplex methods that have been used in endometriosis research, summarized in Table 1 and Figure 1, namely the planar analytical multiplex sandwich ELISA, the multiplex bead-based immunoassay, and the label-based antibody array. We elaborate on each technique and provide a review of the findings that have emerged from prior research on endometriosis and multiplex immunoassays. As the obtained data are highly complex, they should be analyzed with adapted multivariate statistical methods. In the future, data obtained by multiplex immunoassays may be utilized to unravel disease pathology and may lead to improved, timely diagnosis.

3. MULTIPLEX IMMUNOASSAY TECHNIQUES AND THEIR USE IN ENDOMETRIOSIS

3.1. Planar analytical multiplex sandwich ELISA

The planar analytical multiplex sandwich ELISA has been developed from the traditional singleplex ELISA to realize the simultaneous detection of a number of analytes (17). 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 often employed because of the increased sensitivity and broader dynamic range compared to the traditional colorimetric detection (17, 25, 26). A sandwich approach is based on the interaction between a capture antibody for identification purposes, its corresponding antigen, and a detection antibody recognizing the non-overlapping epitopes of the same target protein (Figure 1A) (23).

3.1.1. Endometriosis studies assessing differentially expressed proteins in peritoneal fluid or plasma

In endometriosis, two studies have reported the use of the planar analytical multiplex ELISA, one in PF and the other in plasma (11, 27). A mini-array sandwich ELISA measuring 9 chemokines showed higher concentrations of macrophage inflammatory protein (MIP)-3β in peritoneal fluid (PF) of endometriosis patients, compared with controls suffering of primary infertility (27) (Table 2). Apart from using a multiplex bead-based immunoassay (paragraph 3.2.) and singleplex ELISAs, Vodolazkaia *et al.* performed a planar analytical multiplex ELISA with chemiluminescent detection of osteopontin, insulin-like growth factor-binding protein (IGFBP)-3, and leptin in plasma (11). 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. The resulting panels consisted of cancer antigen (CA)-125, vascular endothelial growth factor (VEGF), Annexin V, and glycodelin or soluble intercellular adhesion molecule (sICAM)-1 (11).

3.1.2. Advantages and disadvantages of the planar multiplex sandwich ELISA

Apart from the possibility to screen for endometriosis biomarkers, multiplex customized panels can be developed, validated, and subsequently implemented in diagnostics (28). Assay miniaturization confers the benefits of reduced incubation times, improved signal-to-noise ratios, and the possibility of high-throughput (18, 19). Due to the sandwich design, specificity and sensitivity are usually good, however this format limits the number of detectable analytes because of the cross-reactivity between antibodies (18). Furthermore, for certain analytes, results obtained by planar multiplex ELISA may differ from those obtained by singleplex ELISAs (29, 30). Assay performance characteristics vary between planar multiplex ELISAs from different manufacturers (coefficient of variation: 2.8.-10%) (31).

3.2. 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 (17, 26, 32). 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 (33). Multiplex bead-based assays have been implemented in different fields of research (34, 35), including endometriosis (11, 36-44). The technique has been used extensively to detect the link between inflammation and endometriosis locally in PF samples (36-40, 42, 43) and to a lesser extent in serum (39, 41, 43, 44) or plasma (11) (Table 3).

3.2.1. Endometriosis studies assessing differentially expressed proteins in peritoneal fluid

Wickiewicz *et al.* showed the significantly elevated expression of IL-10 in PF of endometriosis patients versus controls, alongside IL-6 (40). 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, especially for the subgroup of patients with stage III-IV endometriosis, showing an area under the curve of 0.853 (40). In correspondence with the previous study, Bersinger *et al.* reported the significant upregulation of IL-6 in stage III-IV endometriosis (36). 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) (36). Interferon gamma-induced protein 10 (IP-10) was significantly elevated in the I-IV group, but not in the III-IV subgroup (36). 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 (36). 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 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 (37). Increased PF concentrations of IL-8 and MCP-1 in endometriosis were also reported by Borrelli *et al*., along with increased MIP-3β (42). At the established cut off points, only IL-8 and MIP-3β had the power to statistically predict endometriosis (odds ratios of 5.38 and 3.73, respectively). However, the combined presence of the three chemokines in concentrations above their respective cut offs, rendered a 89.1 % probability of endometriosis (42). An unusual approach was used by Beste *et al.*: unsupervised multivariate analysis using non-negative matrix factorization (NMF) was applied to identify a recurrent pattern of biomarkers (or a consensus signature) in peritoneal fluid of women with pain and/or infertility (38). The consensus signature consisted of 13 differentially expressed, co-varying 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 were indistinguishable from the controls, while the other 13 patients showed four or more elevated "consensus" markers (38). This discrepancy within the endometriosis group may be due to the disease heterogeneity and the dynamic changes of lesion progression (38). While this consensus signature may not be suitable 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 (38). Furthermore the signature may be helpful to identify women who might benefit from post-operative immune treatment (38).

3.2.2. Endometriosis studies assessing differentially expressed proteins in peritoneal fluid and serum

Two studies investigated endometriosis biomarkers in both PF and serum (39, 43). Podgaec *et al.* could not detect any differences in the serum concentration of the five investigated cytokines, but found significantly elevated levels of interferon (IFN)-γ and interleukin (IL)-10 in endometriosis patients in PF (39). Furthermore, the ratios of IL-4/IFN-γ, IL-4/IL-2, IL-10/IFN-γ, and IL-10/IL-2 were significantly different between the endometriosis group and the controls without endometriosis, reflecting a shift towards a Th2 immune response in endometriosis (39). Kocbek *et al.* used multiplex immunoassays and singleplex ELISAs to compare PF and serum samples of patients with ovarian endometriosis to a control group comprising patients with benign ovarian cysts and healthy women (43). In the endometriosis group, PF showed upregulation of glycodelin-A, IL-6, and IL-8, and downregulation of leptin, while in serum only glycodelin-A was upregulated (43). Furthermore, both in PF and serum, two panels of biomarkers were identified using multivariable logistic regression. In PF, the panels consisted of (1) biglycan/leptin ratio, RANTES/IL-6 ratio and age, and (2) ficolin-2/glycodelin-A ratio, IL-8 concentration per mg total protein and age. In serum, the panels consisted of (1) leptin/glycodelin-A ratio and age, and (2) ficolin-2/glycodelin-A ratio and age (43).

3.2.3. Endometriosis studies assessing differentially expressed proteins in serum or plasma

Three articles focused on biomarker discovery in serum or plasma using multiplex bead-based immunoassays (11, 41, 44). Malutan *et al*. showed significantly higher serum concentrations of IL-1β, IL-6, and TNF-α in endometriosis patients versus controls without endometriosis (44). However, at the chosen threshold values, sensitivity of each marker was low (between 50.0 % and 57.1 %) (44). Othman *et al*. found significantly increased concentrations of IL-6, MCP-1, and IFN-γ in serum of endometriosis patients. Using multivariate regression analysis, a panel consisting of IL-6, MCP-1, and IFN-γ showed no improved diagnostic performance compared with IL-6 alone (41). In contrast, multivariate analysis was very instructive in a study published by Vodolazkaia *et al.* (11). This study used, alongside the traditional singleplex ELISAs, two types of multiplex panels: one based on sandwich ELISA (as mentioned in paragraph 3.1.1.) 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 (11). 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 (11).

3.2.4. Advantages and disadvantages of multiplex bead-based immunoassays

The advantage of the bead-based method is the high level of multiplexing and the flexibility in array composition (32). The result is generated from the read-out of multiple beads, where each bead acts as a duplicate, which is favorable for the accuracy and robustness of the assay (24). Furthermore, the advantage of a solution-based bead system is the increased efficiency to capture antigens (34) and the broader dynamic range (17, 34). Generally, good correlation between multiplex bead-based immunoassays and singleplex ELISAs can be found (34), but absolute concentrations tend to diverge unless identical antibodies and reagents are used (22). Assay results may vary between kits from different manufacturers, but this can often be attributed to differences in antibody pairs, antigens for generating the standard curves, and composition of sample diluents and assay buffers (45). Quantitative comparison of one cytokine across different studies warrants caution because of varying robustness among the multiplex bead-based immunoassays (22, 40).

When comparing the two most frequently used multiplex systems in endometriosis, Bio-Plex (Bio-Rad, Hercules, CA, USA) can measure up to 500 analytes in one run, while the maximum number of analytes measured for the Cytometric Bead Array (CBA, BD Biosciences, San Jose, CA, USA) 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 (22). 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 (22). 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 (41).

3.3. 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 3.1., however the label-based antibody array technology is suited for a proteomic approach (16), which is defined by the systematic analysis of proteins in biological samples (46). It is particularly useful as a marker screening tool. This method allows the rapid parallel detection of proteins in a low sample volume (47). However, the sample throughput is usually lower than with the previously discussed methods (paragraphs 3.1. and 3.2.), due to a generally higher cost of these assays (47). An antibody array may be sandwich based (47) as described in paragraph 3.1., but cross-reactivity between antibodies becomes a problem proportional to the size of the multiplex (23). 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 (23) (Figure 1C). As there is no need for an antibody pair, more targets can be included (9). 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 (23). Alternatively, chemiluminescent detection is possible (48). The advantage of the biotin label is that small molecules are less disruptive to the labeled protein than fluorophore tags (9), the possibility for signal amplification and the efficient labeling process, thereby contributing to an increased detection sensitivity (23). 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 (9, 49). 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 (18, 23). Detection sensitivity depends from array to array (23). For cancer research, the use of label-based antibody microarrays has been described in serum and plasma (18, 50).

3.3.1. Endometriosis studies assessing differentially expressed proteins in peritoneal fluid

To our knowledge, in endometriosis research, the use of large scale label-based antibody arrays has been limited to one study published by Hou *et al.* (51) (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 (51). 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 (51). 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 (51). However, since the results were obtained from pooled patient samples, no advanced statistical analysis was performed (51).

3.3.2. Advantages and disadvantages of the label-based antibody array

Advantages of the label-based antibody array approach include the fast identification of a multitude of proteins and the possibility for a quick validation using ELISA (23). Disadvantages of this method include the reduced specificity and the assembly of multiprotein complexes, which may generate a strong disproportionate signal (16). The complexity of human

biological samples prevents the optimal detection of all proteins due to the large concentration range (pg/ml-μg/ml) (18). Furthermore, assays results are usually semi-quantitative and need to be validated with ELISA.

4. MULTIVARIATE STATISTICAL ANALYSIS: LOGISTIC REGRESSION (LR), LEAST SQUARE SUPPORT VECTOR MACHINES (LS-SVM), CLASSIFICATION AND REGRESSION TREE (CART), AND ARTIFICIAL NEURAL NETWORKS (ANN)

As data obtained by multiplex immunoassays can be highly complex, they should be analyzed with adapted statistical methods. 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 (52, 53), as well as least square support vector machines (LS-SVM) (52, 54).

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 (55). 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) (55). In ordinary regression, least squares are used for finding the best fit (56, 57). 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 (55). 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 (58), 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) (52, 54). Both of them are members of the class of kernel methods. They are based on the principle 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. However, this requires carefully tuning the parameter sigma (σ) and the regularization parameter γ to avoid overfitting (59).

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 (60). 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*. (11) 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 (61). 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 (11). Stratifying data into a training and test set is essential for testing the robustness of the models as described in Vodolazkaia *et al*. (11).

Seeber *et al*. (10, 62) used Classification and Regression Tree (CART) to classify endometriosis. CART analysis, which is a nonparametric statistical method, analyzes all possible splits on each explanatory variables and selects the variable and cut off value that identify the best splits which best classifies the subjects with regard to the endometriosis status. The tree can be "learned" by analyzing the splits for each explanatory variables, and the split that maximizes the homogeneity of the cluster with

respect to the endometriosis status is selected. CART creates from each parent note 2 child notes and continues growing the tree, measuring the ability of the explanatory variables to classify subjects.

Artificial neural networks (ANNs), as another advanced method for classification, have been used successively in many biomedical research questions (63, 64) including endometriosis research (65). ANNs is able to recognize complex patterns by learning from a set of data points (known as training examples) to infer rules for the task at hand such as classification.

For the adequate interpretation of data acquired by multiplex immunoassay methods, these multivariate statistical methods should be adopted in future endometriosis studies.

5. CONCLUSION

Due to the increased awareness that multiple markers may be necessary to understand a complex disease such as endometriosis, the simultaneous measurement of different proteins in one sample is emerging as an important tool in endometriosis research and other research areas such as cancer. Multiplex methods are more attractive than the classical singleplex ELISAs, due to the cost-effectiveness and lower volume requirements of precious biological samples (17). They are more user-friendly, quicker, and more sensitive than mass spectrometry methods (18).

Five studies reviewed herein investigated multiplex immunoassays in plasma/serum of women with and without endometriosis, of which four focused on finding diagnostic biomarkers or biomarker panels (11, 41, 43, 44) (Tables 2-3). IL-6 was the only marker to be upregulated in endometriosis in two different reports (41, 44). Discovered panels exhibited only minimal overlap in proteins (11, 41, 43). Remarkably, in two studies that applied both singleplex ELISA and multiplex technologies, the final panels with best accuracy consisted mostly of proteins investigated by singleplex ELISA (11, 43). This may be due to the fact that, in contrast to the singleplex ELISAs in these studies, the multiplex assays consisted mostly of cytokines (11, 43). Measurement of circulating cytokines is complex due to their short half-life (66) and the influence of sample collection methods, storage time, circadian patterns, exercise, and stress (67). Moreover, the relevance of inflammatory cytokines in the discrimination of women with endometriosis from women with non-endometriotic pelvic pathology remains unclear (11). Future studies in peripheral blood should verify the value of multiplex immunoassay panels for biomarker discovery in endometriosis. Large antibody array screening beyond the scope of cytokine measurement could provide new diagnostic insights.

Nine endometriosis studies have applied multiplex panels on peritoneal fluid (PF) samples (27, 36-40, 42, 43, 51) (Tables 2-3). Of those, four reports proposed several different biomarkers or biomarker panels to improve the diagnosis of endometriosis (36, 40, 42, 43). The remaining five studies aimed to elucidate the molecular mechanism behind the pathology of endometriosis. (27, 37-39, 51). A shift to a Th2 immune response in endometriosis was postulated (37, 39), along with a role for chemokines (27), innate inflammatory responses mediated by macrophages (38) and complex cytokine networks (51). Agreement between multiplex studies was the highest for the pro-inflammatory cytokine IL-6 (36-38, 40, 43, 51) and the angiogenic cytokine IL-8 (36-38, 42, 43, 51) as they were both significantly upregulated in PF of women with endometriosis in four of six studies. Both cytokines have been proposed as good PF endometriosis markers in reports employing singleplex methods (66, 68). MIP-3β exhibited a 100% concordance rate in the studies reviewed herein, but this chemokine was only investigated on two occasions with multiplex techniques (27, 42). Elevated levels of IL-6, IL-8, and MIP-3β in PF may indicate the importance of local macrophage activating factors in the pathogenesis of endometriosis (66). The absolute mean/median values tended to diverge widely between studies. Variability between assays is not uncommon in singleplex ELISAs either (69) and may be attributed to varying techniques and/or patient inclusion criteria (66). Cytokine concentrations may also be affected by cyclic changes in PF volume (70).

Despite the relatively small body of work on multiplex immunoassays in endometriosis, the multiplex techniques have contributed to theories on the pathogenesis of endometriosis and have been involved in diagnostic model building for endometriosis. Importantly, the multitude of the data generated from multiplex immunological assays offers unique opportunities to address the complexity of endometriosis. Multiplex analysis of proteins in peritoneal fluid has the potential to provide novel insights into the pathogenesis of endometriosis by identification of co-varying molecules within a network (38, 51), whereas multiplex measurements in peripheral blood could enable identification of a set of biomarkers for diagnostic purposes. However, it is important to note that other promising diagnostic options cannot be explored using multiplex methods, such as the semi-invasive diagnosis of endometriosis by measuring nerve fiber density in eutopic endometrium (13). Furthermore, we emphasize that researchers have struggled to validate previous findings, to achieve sensitivities/specificities that would be acceptable in a clinical context, and to find a marker that rules out patients with a differential diagnosis. This failure to produce a biomarker panel is partly due to use of univariate statistical analysis and the limited number of investigated biomarkers per study, but is likewise the consequence of inadequate selection of controls, unclear patient characterization, and the lack of validation in independent patient cohorts (5). Careful attention to sample collection, study design, assay reproducibility and statistical analysis is warranted (17).Translation of biomarker concentrations into diagnostic models should be achieved by multivariate statistical analysis. Only 6 of the 12 endometriosis studies reported in this review employed multivariate statistical analysis (see Tables 2-4). Samples should be investigated in a multiplex manner using large sample sizes with well-defined patient characteristics (5). Further studies in well-defined patient sets and using adequate statistical models should allow researchers to shed light on the

complex molecular basis of endometriosis by investigating the dynamic interplay among simultaneously measured analytes. Diagnostic panels should be validated in independent patient cohorts before being applied in the clinic. In conclusion, we state that combining multiplex arrays with multivariate statistical analysis holds great promise for understanding and possibly diagnosing endometriosis.

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Abbreviations: ANN: Artificial neural network, 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, GROa: 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

Key Words: Endometriosis, Multiplex immunoassay, Planar analytical sandwich ELISA, Bead-based immunoassay, Label-based antibody array, Biomarker, Multivariate statistics, Review

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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 (biotinylation)
Required antibodies	Antibody pairs (sandwich format)	Antibody pairs (sandwich format)	Capture antibody only
Format	96-well plate or microarray	96-well plate	Microarray
Method of detection	Chemiluminescence, fluorescence	Fluorescence (flow cytometry)	Chemiluminescence, fluorescence (laser scanner)
Application possible in clinical diagnostics	Yes	Yes	No
References in endometriosis research	(11, 27)	(11, 36-44)	(51)
Cost	Generally lower cost per sample and per analyte than traditional ELISA, depending on the amount of analyzed markers.	Generally lower cost per sample and per analyte than traditional ELISA, depending on the amount of analyzed markers.	High cost per sample Low cost per analyte

Table 2. Overview of endometriosis studies using the multiplex sandwich-ELISA

Reference	**Sample type**	**Type of assay (company)**	**# analytes**	**Sample size**	**Multivariate statistics**	**Findings**
Laudanski *et al*., 2006 (27)	PF	Array spotted in 3 x 3 pattern using the Biochip Arrayer I™ (Packard Instruments) (71)	9	Endo *n* = 24 Ctrl *n* = 18	No	↑ MIP-3β
Vodolazkaia *et al*., 2012 (11)	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 were not included in the final panel for US negative endometriosis which consisted of: CA-125*, Annexin V*, VEGF and glycodelin* or sICAM-1*

PF = Peritoneal fluid. Endo = endometriosis group. Ctrl = control group. ^ = subset of ultrasound-negative endometriosis patients. * = measured by singleplex methods

Table 3. Overview of endometriosis studies using the multiplex bead-based immunoassay.

Reference	Sample type	Type of assay (company)	# analytes	Sample size	Multivariate statistics	Findings
Podgaec *et al*., 2007 (39)	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
Othman Eel *et al*., 2008 (41)	Serum	Bio-Plex Protein Array System (Bio-Rad)	9	Endo *n* = 68 Ctrl *n* = 70	Yes (regression analysis	Panel: MCP-1, IL-6, IFN-γ
Mier-Cabrera *et al*., 2011 (37)	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
Bersinger *et al*., 2012 (36)	PF	Bio-Plex platform (Bio-Rad Laboratories), selection from Human Cytokine panels I and II	13 (panel I) + 5 (panel II)	Endo *n* = 101 Ctrl *n* = 32	No	↑ IL-6, IL-18, eotaxin, MCP-1, IP-10
Vodolazkaia *et al*., 2012 (11)	Plasma	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)	Panel: CA-125*, Annexin V*, VEGF and glycodelin* or sICAM-1*
Wickiewicz *et al*., 2013 (40)	PF	Cytometric Bead Array Th1/Th2 Human Cytokine Kit II (BD Biosciences)	6	Endo *n* = 36 Ctrl *n* = 42	No	↑ IL-10, IL-6
Beste *et al*., 2014 (38)	PF	Bio-plex Group I 27-plex, Group II 21-plex (Bio-Rad Laboratories)	27 (group I) + 21 (group II)	Endo *n* = 57 - Progestins *n* = 16 - No med *n* = 41 Ctrl *n* = 20	Yes (NMF)	Consensus signature: IL-8, RANTES, MIF, IL-6, MCP-1, G-CSF, MIG, HGF, IL-10, IL-16, IL-1ra, GROα, IL-1β
Borrelli *et al.*, 2015 (42)	PF	Bio-plex assay: Human Cytokine panel Group I and Human Chemokines Panel (Bio-Rad Laboratories)	3 (group I) + 3 (chemokines panel)	Endo *n* = 36 - Med *n* = 16 Ctrl *n* = 27 - Med *n* = 9	Yes (multiple logistic regression)	Panel: IL-8, MCP-1, MIP-3β
Kocbek *et al*., 2015 (43)	PF, serum	Bio-plex assay: Human Cytokine panel I and II (Bio-Rad Laboratories)	6 (panel I) + 4 (panel II)	Endo *n* = 58 - Med *n* = 25 Ctrl n = 40	Yes (Logistic regression)	Panels in PF: [1] biglycan*/leptin* ratio, RANTES*/IL-6 ratio and age, and [2] ficolin-2*/glycodelin-A* ratio, IL-8 concentration per mg total protein and age Panels in serum: [1] leptin*/glycodelin-A* ratio and age, and [2] ficolin-2*/glycodelin-A* ratio and age
Malutan *et al.*, 2015 (44)	Serum	Human Cytokine 30-Plex Panel (Invitrogen)	6	Endo *n* = 80 Ctrl *n* = 80	No	↑ IL-1β, IL-6, TNF-α

PF = Peritoneal fluid. Endo = endometriosis group. Ctrl = control group. Med = use of hormonal medication. ^ = subset of ultrasound-negative endometriosis patients. * = measured by singleplex methods

Table 4. Overview of endometriosis studies using the label-based antibody array.

Reference	Sample type	Type of assay (company)	# analytes	Sample size	Multivariate statistics	Findings
Hou *et al*., 2009 (51)	PF	Biotin Label-based Human Antibody Array I (RayBiotech)	507	Endo *n* = 6 Ctrl *n* = 3	No	↑ 74 ↓ 4

Endo = endometriosis group. Ctrl = control group

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. Alternatively, fluorescent detection methods can be applied. 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, or alternatively by adding a biotinylated secondary antibody and a streptavidin-conjugated reporter dye. 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 (e.g. biotin), and those labels can be detected by a fluorescence or enzyme conjugated molecule (e.g. streptavidin), eliminating the need for a detection antibody.

Running title: multiplex immunoassays and endometriosis