New horizons in the non-invasive diagnosis of endometriosis

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Summary

Endometriosis is a chronic disorder, clinically associated with chronic pelvic pain, dyspareunia, dysmenorrhea, and infertility. Its socio-economic impact is extensive, given the large number of affected women in reproductive age, its symptomatology (that interferes with normal social life and the patient's ability to work), and its frequent association with infertility. Nonetheless, the diagnosis of endometriosis is still difficult and late in the evolution of the disorder. The authors have used the Quality Assessment of Diagnostic Accuracy Studies (QUADAS) criteria to make a systematic review of the literature of the last 28 years, seeking to identify potential biomarkers useful for a non-invasive diagnosis of endometriosis. The authors have highlighted more than 50 biomarkers in the studies included in the present report, but they have not succeeded in identifying a clinically useful non-invasive diagnostic biomarker or panel of biomarkers. More studies are needed before biomarkers can be introduced in clinical practice.

Key words: Endometriosis; Infertility; Peripheral biomarkers; Early diagnosis**.**

Introduction

Endometriosis is a chronic, estrogen-dependent disorder, characterized by the presence of endometrial glands and stroma in an ectopic site. It is clinically associated with chronic pelvic pain, dyspareunia, dysmenorrhea, and infertility. Endometriosis has a high socio-economic impact given the large number of affected women in reproductive age (10% - 15%); its symptomatology undermines normal family and social life and it interferes with the patient's ability to work. The disorder is frequently associated with infertility. The partial understanding of the pathogenesis, its multifactorial nature, and the low specificity of its symptoms render the diagnosis of endometriosis difficult and late in the evolution of the disorder [1,2].

The scientific literature of recent years has shown a growing interest in the research on biomarkers and sets of biomarkers that could be useful in making an early and non-invasive diagnosis of endometriosis and in following-up treated patients and identifying relapses in their earliest stages.

The goal of the present study was to highlight all the biomarkers (plasma, serum, urinary, peritoneal, and endometrial biomarkers) proposed in the international scientific literature of the last 28 years and, through a meta-analytic reprocessing of the data, assess their clinical value (based on sensitivity and specificity) in making a non-invasive diagnosis of endometriosis.

Materials and Methods

The present work was divided into three stages: computer search throughout the scientific literature on this issue from January 1984 to January 2012, definition of the inclusion and exclusion criteria, analysis of the sensitivity (S), and specificity (Sp) of individual biomarkers and panels of biomarkers proposed by the authors.

The computer search envisaged the use of some online medical search engines (PUBMED, EMBASE, MEDLINE, CINHAL) and of the following keywords: endometriosis, plasma-serum-blood-urine-biological-tissue-endometrial biomarkers, cells, diagnosis, non invasive, and mass screening. Only publications in English that met the *inclusion and exclusion criteria* (Table 1) were taken into account. A further selection was then made using the Quality Assessment of Diagnostic Accuracy Studies (QUADAS) criteria in the version modified by Whiting in 2003 (Table 2). Finally through the statistical processing of the data, the best potential biomarkers or panels of biomarkers (greater specificity and sensitivity) for a non-invasive diagnosis of endometriosis were identified.

Results

The computer search produced 11,665 total results; of these 11,488 were eliminated after evaluating the title, content of the abstract, and compliance with the Quality Assessment of Diagnostic Accuracy Studies inclusion and exclusion criteria" and with the "QUADAS criteria". In this way, a final number of 177 articles remained whose analysis highlighted many potential biomarkers and panels of biomarkers, that are listed below:

Cytokines

Interleukin 6 (*IL-6)*: Six studies show a relationship between increased IL-6 serum levels and endometriosis [3-7]. In particular, in the study by Martinez *et al.* [7] high levels of IL-6 were found above all in women with a Stage I-II disease. With a threshold value of 25.75 pg/ml, a 75% sensitivity, and an 83.3% specificity were obtained. Bedaiwy *et al.* showed a sensitivity and specificity respectively of 90% and 67% with a threshold of two pg/ml [4]. On the contrary, other studies did not report a significant increase in IL-6 [8-12].

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Table 1. — *Inclusion and exclusion criteria of the study.*

Inclusion criteria	Exclusion criteria
• Biomarkers assayed from serum, plasma, urine, peritoneal fluid; • Visual and/or histologic confirmation of endometriosis during laparoscopic exploration	• Biomarkers obtained using invasive procedures • Studies that did not include healthy controls • Studies on CA125 before the meta-analysis by Mol *et al.* (1998); • Studies with male individuals among the controls; • Studies that required extended cell cultures (> 24h) to show differences in the expression of the biomarkers

Table 2. — *Inclusion and exclusion criteria of the study.*

Modified version of the QUADAS criteria (Whiting *et al.*, 2003)
• Were the patients and controls recruited from women with symptoms suggestive of endometriosis?
• Were the selection criteria described clearly?
• Was the time between diagnosis and the assay of the biomarkers sufficiently short to avoid variations in the stage of the disease?
• Was the absence of disease among the controls checked surgically?
• Was a sufficient description made of the method?
• Were the results interpreted using a *blinded fashion model*?
• Was the diagnosis of endometriosis made without knowing the outcome of the test on the biomarkers?
• Were intermediate or non-interpretable results reported?
• Was the decision, if any, to drop out of the study declared?
• Were the samples collected during an adequate phase of the menstrual cycle or were the results correct for the phase of the cycle?
• Were the samples collected from women with a specific stage of the disease or were the results correct for the stage of the disease?

Interleukin 8 (IL-8): A study of 2003 showed increased serum levels especially for Stages I and II [13].

TNF-α: Various authors report particularly high serum and peritoneal levels in women with endometriosis in Stages III and IV [5, 13-18]. In the study by Bedaiwy *et al.*, with a threshold of 15 pg/ml, a sensitivity and specificity of 100%, and 89% [4], respectively, are achieved when the cytokine assay was performed on the peritoneal fluid of affected women.

Monocyte chemotactic protein 1 (MCP-1): by using a threshold value of 100 pg/ml, a 65% sensitivity and a 61% specificity are obtained [19].

Interferon-gamma (IFNγ): In 2003, Darai *et al.* found an increase in the serum levels of IFNγ in women with endometriosis [6].

Other cytokines: Other interesting findings are the high levels of interleukin 1α (IL-1α) in the serum [20] and high levels of IL-12 and IL-18 in the peritoneum fluid of affected women [21-23].

Inflammatory markers

C-reactive protein and high-sensitivity C-reactive protein (CRP and hs-CRP): The study carried out by Lermann shows higher CRP (3.54 mg/l) and higher hs-CRP (3.61 mg/l) average values in the group of patients with endometriosis (E-group), as compared to healthy controls (non-E group) (CRP = 2.88 mg/l; hs-CRP = 2.48 mg/l) [24]. Although there is a real difference in the concentration of molecules between the two study groups, the difference is not statistically significant. Hence, CRP and hs-CRP cannot be potential biomarkers.

Antibodies (Ab)

Anti-endometrium antibodies: These have an 86% sensitivity and a 76% specificity in the diagnosis of endometriosis [25]. Sensitivity and specificity increase considerably, up to 87%, if used for the diagnosis in women with infertility, dysmenorrhea, and chronic pelvic pain [26]. IgG antibodies are those that appear to correlate most with endometriosis [27,28]. A recent study identified eight new antibodies against some endometrial antigens such as: tropomyosin 3 (TPM3), stomatin-like protein 2, (SLP2), and tropomodulin-3 (TMOD3). The following are respectively, the sensitivity and specificity of these antibodies in the early stages of the disease: Ac anti-TPM3a (61%, 93%), Ac anti-TPM3c (44%, 93%), Ac anti-TMP3d (78%, 89%), Ac anti-SLP2a (50%, 96%), Ac anti-SLP2c (61%, 93%), Ac anti-TMOD 3b (61%, 96%), Ac anti-TMOD3c (78%,93%), Ac anti-TMOD3d (78%, 96%) [29].

Anti-carbonic anhydrase Ab: Kiechle *et al*. have shown a sensitivity of 13% for type I and of 24% for type II [30].

Anti-transferrin and anti-α2-HS glycoprotein Ab: these present maximum sensitivity and specificity if assayed using the ELISA technique, reach values of 95% [31, 32].

Ab against oxidative stress markers: women with endometriosis present increased levels of Ac anti-lipid peroxide modified rabbit serum albumin, Ac anti-copper oxidized low-density lipoprotein, and Ac anti-malondialdehyde-modified low density lipoprotein [33].

Anti-laminin Ab: some authors have found high concentrations of these autoantibodies in patients with infertility (the cut-off of one U/ml has a sensitivity of 43% and a specificity of 89%) [34, 35].

Anti-α enolase Ab: have a sensitivity and specificity comparable with that of CA125 [36].

Anti-PDIK1L (PD-interacting kinase 1 like) Ab: PDIK1L is abundantly expressed by endometriotic cells. With a cut-off of 300 U/ml, the test provides a sensitivity of 59.4% and a specificity of 84.1%. Anti-PDIK1L autoantibodies are expressed in larger amounts in Stage I-II, therefore they could be of assistance in the early diagnosis of the disease [36].

Anti-syntaxin 5 Ab: at a cut-off of 400 U/ml shows a sensitivity of 53.6% and a specificity of 87.8% in a Stage II endometriosis [37].

Anti-IGFII mRNA-binding protein1 (IMP1) and Anti-cyclin B1 Ab: Yi *et al.* have reported for IMP1 a sensitivity of 85.7% and a specificity of 63.3% in women with en-

dometriomas. In combination with cyclin B2, it presents lower sensitivity (83.9%) but greater specificity (72.7%) [38].

Glycoproteins

Cancer antigen-125 (CA-125): This is the glycoprotein of great interest for endometriosis. Some recent studies show that CA125 is the most reliable glycoprotein in diagnosing Stage III-IV endometriosis [39, 40]. Xavier *et al.* show that the cut-off that provides the greatest sensitivity and specificity (86% and 89% respectively) is lower (22.6 IU/ml) than that reported in most of the literature (35 IU/ml) [41]. Various studies have established that the serum concentration of CA125 correlates with the severity of the disease [42] and tends to be higher in women with ovarian endometriosis (with a threshold of 30 IU/ml the sensitivity is 79% in women with endometrioma and drops to 44% for other sites) [43]. Finally, O' Brien *et al.*, have demonstrated that the technique used to assay CA- 125 considerably influences its efficacy as clinical biomarker of endometriosis [44].

Cancer antigen-19-9 (CA-19-9): The threshold value of 5.4 IU/ml gives the best diagnostic performance [45, 46].

Cancer antigen-15-3 (CA-15-3) and Cancer antigen-72 (CA-72): Various authors have studied these glycoproteins but have obtained contrasting results [47-49].

Haptoglobine: Typically produced by endometriosis lesions. A selective increase in serum levels of the β isoform in the follicular phase of the menstrual cycle has been found [50].

Follistatin: The serum concentrations of follistatin are raised in women with endometriomas compared to healthy controls [51].

Gremlin-1: This glycoprotein is hyperexpressed in the endometrial stroma of affected women. Its serum concentration is found to be increased exclusively in the proliferating stage [52, 53].

Cell populations

The patients with endometriosis present alterations in the normal lymphocyte count and in the monocyte-macrophage line. In particular the following is observed: increase in T suppressor lymphocytes (CD8+, CD11+) and in activated T lymphocytes (CD3+ ed HLA-DR+) [54,55], reduction in the circulating NK cells [56,57], and increase in the neutrophil/lymphocyte ratio (NLR) (consequence of the increase in circulating neutrophils) [58].

Other immunological biomarkers

Endometriosis is associated with an increase in the serum concentrations of the C3 and C4 complement fractions [59] and in the soluble forms of CD4 and CD23 [60-62]. A recent paper has shown the presence of high levels of peptides known as human neutrophil peptides 1, 2, 3 (HNP 1-3) in the peritoneal fluid of affected women [63].

Adhesion molecules

From the studies, the present authors have examined that it can be inferred that endometriosis is associated with an increase in the serum concentrations of the following adhesion molecules: ICAM-1 (particularly high in Stages I-II of the disease [64, 65], VCAM [66], E-cadherin (that does not present any particular correlation with the stage of the disease) [67], and finally, osteopontin [68].

Growth factors

A study has shown an increase in the serum levels of IGF-1 exclusively in Stages III-IV [69].

Circulating cell-free DNA (ccf-DNA)

Through real time PCR, it was possible to demonstrate a ccf-n DNA plasma concentration that was significantly greater in patients with endometriosis compared to controls; the test presents a sensitivity of 70% and a specificity of 87% [70].

Hormones

Prolactin (PRL): The association of hyperprolactinemia, galactorrhea with endometriosis, has been known for more than 30 years. Recent studies have shown the presence of hyperprolactinemia (PRL > 20 ng/ml) in 30% of women with endometriosis and infertility, whereas none of the fertile women with endometriosis and none of the controls presented raised levels of this hormone [71].

Luteinizing hormone (LH), testosterone, cortisol: Various studies have shown increased serum levels of this hormone in women with endometriosis; testosterone seems to be selectively associated with ovarian endometriosis and cortisol with advanced stage endometriosis (III-IV) [71, 72].

Leptin and adiponectin: Their serum levels are respectively increased and reduced in patients with endometriosis compared to controls [73-75].

Angiogenetic factors

Various studies have demonstrated the increase, in the advanced stages of this disorder, in serum concentrations of VEGF, and in one of its soluble receptors (sFlt-1) present in the serum and in the urine [18, 19, 76], Angiogenin [77], in FGF-2 [78] and finally in HGF [79].

Proteomic markers

The analysis of protein expression profiles in the serum and in the endometrium of women with the disorder is one of the most promising areas of research on potential biomarkers: the presence, absence, hypo- or hyper-expression of peculiar isoforms in the blood and/or endometrial tissue, could indicate new useful biomarkers. The protein peaks found, indeed, could be used to construct a diagnostic protein pattern in patients with endometriosis. The most important proteomic studies carried out so far are the following: Wang *et al.* [80] who have identified a pattern consisting of five protein peaks endowed with a sensitivity and specificity equal to 92% and 90%, respectively; the study by Kyama *et al.* have used two proteomic panels : the first, that examined endometriosis of Stages I-II, presented a sensitivity and specificity of 100%, and the second panel showed a sensitivity of 80% and a specificity of

70%. Furthermore, this latter study developed a protein panel suited to the diagnosis of endometriosis irrespective of the stage of the disorder that consists of five protein bands and presents a sensitivity of 89.5% and a specificity of 90% [81].

Other potential biomarkers

Serum urocortin: it presents considerably increased values in the ovaries of women with endometriosis; it is therefore useful in making a differential diagnosis of the ovarian mass Sensitivity is 88%, and specificity is 90% [82]. In actual fact, a more recent study showed lower values: 72.6% sensitivity and 45.7% specificity [83].

Protein PP14: high especially in advanced stages [84];

Tumor associated trypsin inhibitor (TATI): sensitivity 34% and specificity 85% [85];

Amyloid A: increases in Stages III-IV;

Paroxonase 1 (PON-1): antioxidant glycoprotein. Its sensitivity is 98% and its specificity is 83% [86];

Matrix metalloproteinase 9 and 2 (MMP-9, MMP-2) and phosphatase of regenerating liver 3 (PRL-3): reach a sensitivity of 87.5% in Stages III-IV [87,88].

Urinary vitamin D-binding protein (VDBP): sensitivity 58%, specificity 76% [89].

Urinary cytokeratin-19 (CK-19): initial studies have established a sensitivity and a specificity of 100% [90].

Panels

From the statistical analysis of the panels of biomarkers proposed in the literature of the last 28 years, those with greater diagnostic efficacy are:

IL-6, IL-8, TNFα, hs-CRP, CA-125, CA19-9 (sensitivity = 92.2% specificity = 82%) [91];

CA-125, NLR: (sensitivity > 86% specificity > 89%) [58];

PGP9, VIP, substance P (sensitivity = 95% e specificity = 100%) [92];

CCR1 m RNA, MCP1, CA-125 (sensitivity = 92.2% specificity = 82%) [93].

CA-125, CA19-9, survivin: (sensitivity = 87%) [94].

Discussion

The numerous difficulties encountered in pursuing the present objective are linked to various factors. First of all, a negative impact was due to the inherent characteristics of endometriosis such as: its multifactorial nature and the heterogeneity in terms of stage, site, and aspect of the lesions. Moreover, specific characteristics found in the various studies have proven to be important such as: inadequate patient sample (insufficient number, lack of confirmation of the diagnosis of endometriosis through laparoscopic exploration, lack of definition of recruitment criteria), and/or inadequacy of the group of healthy controls (limited number, not well-defined recruitment criteria, presence of co-morbidities); poor specificity of most of the biomarkers taken into account; the frequent disagreement among the data provided by various studies on the same biomarker (attributable to: method used, threshold value, timing of the sampling of the biological samples, adjustment of data to menstrual phase), and the lack of publication of studies with negative or irrelevant outcomes that could have provided useful insight [95].

With regards to the biomarkers, some of them, albeit presenting high sensitivity, do not have an adequate level of specificity, since they are implied also in physiological processes (cytokines) or in various pathologies. Some examples of biomarkers having low specificity are: CA-125 glycoproteins CA-19-9 [96], urinary IGF [97], VEFG and anti-cardiolipin antibodies [98], urocortin [99]. The diagnostic efficacy of biomarkers is considerably increased by the phases of the menstrual cycle, by the stage of the disease, and by the site of the lesions: elements that can cause conspicuous variations in terms of sensitivity and specificity of the values. The design of the studies the present authors selected is an important factor in evaluating the reliability of the results obtained. Indeed, even though rigid inclusion criteria were used, many works concerning the same biomarker often provided diverging results because of the wide variability in the threshold value taken into account, in the method used for the assay of the biomarkers, in the origin of the biological sample, in the method, timing of sampling and storage of the biological sample, in the selection criteria of the group of patients and controls and the breadth and scope of the results, and finally the statistical instrument used for processing the results.

The threshold selected significantly affects the diagnostic accuracy of the biomarkers; this is the case of CA-125 whose sensitivity ranges from 27% to 79% depending on the cut-off that was adopted [41-44], and of IL-6 with a sensitivity varying between 71% and 90% and a specificity ranging from 51% to 89% [4,7,8,12]. Of considerable importance is also the biological sample, as regards the type of sample and the sampling and storage techniques. TNF-α [5] has a sensitivity and specificity of about 95% when serum assays are performed, and a sensitivity of 100% and specificity of about 89% when assays are performed on the peritoneal fluid. An adequate selection of the group of patients and controls is indispensable for the quality of the study. In many studies the control group was not adequately selected:

Indeed a fundamental factor is the heterogeneity of the control groups that should include healthy individuals as well as women with symptoms suggestive of endometriosis in whom however the disease has not been excluded with a laparoscopic test. At the same time, with reference to the studies that included among the controls women with benign gynaecological disorders, one cannot exclude that the pathologic condition of some women may have affected the outcome of the study. The handling difficulties instead are a limit to the application of the promising proteomic tests in clinical practice. Indeed it would be a good thing to be able to purify and identify protein molecules corresponding to the protein peaks, so as to introduce immunological tests that assay these proteins in the laboratory without necessarily having to use the SELDI-TOF-MS techniques.

Conclusion

At this point in time, endometriosis is a disorder with a high socio-economic impact whose diagnosis is made difficult by the poor knowledge of its etiopathogenesis, by the non-specificity of its symptoms, and by the lack of an effective non-invasive test. The aim of this study was to search for a biomarker or a panel of biomarkers with sensitivity, specificity, and ease of use suited to make a non-invasive diagnosis of endometriosis. Unfortunately, the present research data were not sufficient to identify a totally reliable non-invasive diagnostic protocol that could be immediately introduced into clinical practice, especially for the lack of very high quality studies, for the large discrepancy between the results of different studies carried out on the same biomarker, for the absence at the present time of a molecule or a panel of molecules that are exclusively correlated to the endometriotic disorder, and finally, for the difficult handling and/or costs of some tests.

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