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Prototype of Multiplex Bead Assay for Quantification of Three Serum Biomarkers for In Vitro Diagnosis of Endometriosis

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Endometriosis is a very common disease, affecting 10% of women in the reproductive age. To date, a significant delay between onset of the symptoms and definitive diagnosis is caused by the lack of a reliable non-invasive diagnostic test. Recently, the potential value as diagnostic markers for endometriosis of three proteins (Zn-alpha2-glycoprotein, serum albumin, and complement C3 precursor), has been showed. In this article, we have defined the experimental conditions for the development of a multiplex bead array assay for rapid and simultaneous quantification of these three biomarkers in the serum of patients with endometriosis. Finally, pivotal experiments on a small cohort of patients have confirmed the diagnostic value of this assay.

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Endometriosis is a pathological condition characterized by the presence of endometrium-like tissue outside of the uterine cavity (Baldi et al., 2008). It is a very common gynecological disease, that affects up to 10% of reproductive-aged women (Bulun, 2009). The most well known and characterized localization of the ectopic endometrium is on the pelvic peritoneum and ovaries, but not less important localization is under the peritoneal surface. Indeed, this last form of endometriosis, the so-called deep-penetrating endometriosis, is associated with more severe symptoms (Signorile et al., 2009a). It must be underlined the fact that this disease causes substantial morbidity, and infertility, and that the necessary extensive medical and surgical treatments are associated with significant costs and risks (Fuldeore et al., 2011). Hence, endometriosis has important socio-economic implications. Surprisingly, despite the fact to be a very common disease, the pathogenesis of endometriosis is still unknown. Up to day, retrograde menstruation and coelomic metaplasia are the most recognized hypotheses (Giudice and Kao, 2004; Benagiano and Brosens, 2006). Our research group has recently produced evidences, both in vitro and in vivo, supporting the idea that endometriosis is caused by fine-tuning alterations of the female genital system development during the foetal life (Signorile et al., 2009b, 2010b, 2010c, 2012; Signorile and Baldi, 2010a; Crispi et al., 2013). The consequences of these observations have, potentially, a great impact on the pathogenesis of endometriosis and the scientific community is still debating on them (Signorile and Baldi, 2015a).

As a matter of fact, endometriosis is still an under- and under-treated disease, being the symptoms mostly not-specific, and all the non-invasive approaches such as blood tests, ultrasound, and magnetic resonance imaging have no sufficient power for a definitive diagnosis of endometriosis (Ballard et al., 2006; Signorile and Baldi, 2015b). In the great majority of the cases, the definitive diagnosis is reached only by histological examination of the ectopic implants following invasive surgical procedures (Bulun, 2009). As a consequence of this, the estimated time interval between insurgence of the disease and definitive diagnosis is very long and consists of 8–12 years (Hadfield et al., 1996). Leaving from this background, it is evident that the definition of reliable blood tests for the non-invasive diagnosis of endometriosis would be of great value in clinical practice. Such tests could allow to recognize with a significant shortening of time patients with endometriosis among all the women with sub-fertility with or without pains and to identify the patients that could take advantage from laparoscopic surgery, a procedure that has been reported to decrease pain and increase fertility (D'Hooghe et al., 2006). Despite the fact that a significant number of studies have been performed to identify biomarkers for the diagnosis of endometriosis, still there are not reliable blood tests (May et al., 2010) and none of the suggested markers is actually recommended in routine clinical care (Mihalyi et al., 2010; Fassbender et al., 2012; Gajbhiye et al., 2012).

Our research group, using a proteomic approach based on 2DE-gel analysis, has recently identified three novel potential biomarkers for the diagnosis of endometriosis: Zn-alpha2-glycoprotein (AZGP1), human serum albumin (HSA), and complement C3 precursor (Signorile and Baldi, 2014, 2015c). Indeed, these three proteins were differentially expressed in a cohort of endometriosis patients respect to a group of healthy women, using an enzyme-linked immune-assay (ELISA). Appropriate statistical analyses, have demonstrated the diagnostic value of these biomarkers.

In this study, we propose to define a prototype of multiplex in vitro diagnostic assay by means of the Luminex technology to define a non-invasive assay for simultaneous quantification of these three proteins in the serum. Indeed, the Luminex system is based on the creation of 100 color-coded fluorescent bead sets, each of which can be conjugated with a unique specific

Abbreviations: 2DE, two dimensional gel electrophoresis; AZGP1, Zn-alpha2-glycoprotein; ELISA, enzyme-linked immunosorbent assay.; HAS, human serum albumin.

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reactant, such as an antibody. As a consequence, up to 100 unique analytes can be quantified in a single well of a 96-well plate (Lynch et al., 2014). This multiplex approach has become widely used in the last years for high-throughput analysis of small volume samples and its value both in basic science and in preclinical and clinical trials is recognized by the scientific community (Dunbar, 2006; Bai et al., 2015). In this article, the experimental conditions for this novel Luminex bead array for AZGP1, HSA, and complement C3 precursor were defined and the potential diagnostic value of the array was investigated on a small cohort of endometriosis patients.

Materials and Methods

Study population

Twenty endometriosis patients and three healthy controls were recruited by the Centro Italiano Endometriosi, Rome. The study was approved by the ethical committee of the Fondazione Italiana Endometriosi. All the subjects signed a written informed consent. In Table 1, the clinical characteristics of the patients enrolled are depicted.

The diagnosis of endometriosis was confirmed by laparoscopy and histology in all patients. Moreover, the stage of endometriosis was categorized according to the Revised American Society for Reproductive Medicine classification of endometriosis (Revised American Society for Reproductive Medicine classification of endometriosis, 1997). Subjects in the control group were women in reproductive age regularly cycling and spontaneously ovulating. Patients using hormonal medication and patients operated within 6 months before the time of sample collections were excluded.

Sample collection and serum preparation

The peripheral blood samples (10 ml) were collected by venipuncture. Sera were centrifuged at 3.000 rpm for 10 min at 4°C, separated in a tube, labeled, and aliquoted at −80°C. Hemolyzed samples were discarded from the study protocol.

Coupling protocols

The stock uncoupled microsphere suspension was resuspended and transferred in $1{,}25 \times 10^6$ COOH-magnetic beads Bio-Rad, Hercules, CA (100 μl solution). The tube was placed into a magnetic separator (Magnetic separator Bio-Rad [Catalog #17102010]) for 30 sec, then the supernatant was removed. Microspheres were resuspended in 100 μl of beads wash buffer (PBS 0.1M 0.02% tween) and vortexed for 20 sec. The washed microspheres were resuspended in 80 μl of beads activation buffer (0.1M NaH_2PO_4 pH 6.2). About 10 μl of EDC (N-(3-dimethylaminopropyl)-N′-ethyl-carbodiimide hydrochloride) 50 mg/ml and 10 μl of NHSS (N-hydroxysulfosuccinimide sodium salt) 50 mg/ml were added, vortexed for 20 sec and incubated in dark for 20 min at room temperature with gentle mixing. The tube was placed into a magnetic separator for 30 sec. Then, the supernatant was removed, and 150 μl PBS 1× was added and vortexed for 20 sec. Again, magnetic separation was performed for 30 sec, and the supernatant was removed. Then, either 500 μl anti-HSA antibody (Abcam, Cambridge, UK, ab18079, mouse monoclonal 1.B.732) 80 μg/ml in PBS, or 500 μl anti-AZGP1 antibody (Sigma–Aldrich SRL, Milano, Italy, SAB1411103 rabbit polyclonal) 40 μg/ml in PBS, or 250 μl anti-C3antibody (Abcam ab36989, mouse monoclonal 10A1) in PBS 80 μg/ml were added and incubated in dark for 120 min at room temperature with gentle mixing. Magnetic separation was performed for 30 sec and the supernatant removed. About 250 μl blocking/storage buffer (BSA 1% 0.05% sodium azide in PBS 0.1M pH 7.4) was added, and incubated in dark for 30 min at room temperature with gentle mixing. Then, magnetic separation was performed for 30 sec and the supernatant removed. About 500 μl of blocking/storage buffer was added and vortexed for 20 sec. Magnetic separation was performed for 30 sec and the supernatant removed. Finally, 150 μl storage buffer was added and the coupled beads stored at 4°C in dark. To note, in the coupling protocol for anti-C3 antibody a further step of incubation with G Protein from *Streptococcus* sp. (Sigma–Aldrich) 40 μg/ml in PBS was included before the incubation with the antibody.

TABLE 1. Clinical characteristics of the 20 endometriosis patients

Median age	Stage of the disease	Cycle phase
32	10 pts stage IV 5 pts stage III 4 pts stage II 1 pts stage I	12 pts secretory 6 pts proliferative 1 pts menstrual

Pts, patients.

Set up of a Luminex quantitative prototype for the detection of HSA, C3 protein, and AZGP1

1-Plex protocol. The appropriate antibody-coupled microsphere set was selected. Then, 50 μl/well of bead diluted 1:100 in PBS-BSA 1% 0.05% sodium azide (PBS-BN) were added, followed by either 50 μl/well HSA-Biotin-TB (8 μg/well) in competition with different concentration of native protein (Abcam ab7473 Human Serum Albumin full length protein from 5 to 0.005 μg/well), or 50 μl/well of C3-Biotin-TB (100 ng/well) in competition with different concentration of native protein (Abcam ab167802 Human C3 full length protein from 50 to 0,04 ng/well), or 50 μL/well AZGP1-Biotin-TB (200 ng/well) in competition with different concentration of native protein (Abcam ab151916 Human Zinc Alpha 2 Glycoprotein full length protein from 100 to 0.01 ng/well), or in competition with serum diluted 1:1000 in PBS-BN for Albumin and C3 and serum diluted 1:4 in PBS-BN for AZGP1. The plates were covered and incubated for 60 min at room temperature on a plate shaker. Then, magnetic separation was performed for 30 sec. The plate was washed with 3 × 100 μl of PBS-BN, and 50 μl/well Streptavidin-Phycoerythrin (SAPE) 4 μg/ml in PBS-BN was added. The plate was covered and incubated for 30 min at room temperature on a plate shaker. Magnetic separation was performed for 30 sec and plate was washed with 3 × 100 μl of PBS-BN. Microspheres were resuspended in 100 μl/well PBS-BN and analyzed 50 μl on the Luminex analyzer according to the system manual.

2-Plex protocol. HSA/C3. The appropriate antibody-coupled microsphere sets were selected. Beads were diluted 1:200 in PBS–BSA 1% 0.05% sodium azide (PBS-BN), 50 μl/well. About 50 μl/well HSA-Biotin-TB (8 μg/well) and C3-Biotin-TB (100 ng/well) in competition with different concentration of native protein (HSA from 5 to 0.005 μg/well, C3 from 50 to 0.04 ng/well), or in competition with serum 1:1000 finale in PBS-BN were added. The plate was covered and incubated 60 min at room temperature on a plate shaker. Magnetic separation was performed for 30 sec and the plate washed with 3 × 100 μl of PBS-BN. 50 μl/well SAPE 4 μg/ml in PBS-BN was added, the plate covered and incubated for 30 min at room temperature on a plate shaker. Magnetic separation was performed for 30 sec and the plate washed with 3 × 100 μl of PBS-BN. Microspheres were resuspended in 100 μl/well PBS-BN and analyzed 50 μl on the Luminex analyzer according to the system manual.

3-Plex protocol (standard only). HSA/C3/AZGP1. The appropriate antibody-coupled microsphere sets were selected. Beads were diluted 1:200 in PBS-BSA 1% 0.05% PBS-BN, 50 μl/well. About 50 μl/well HSA-Biotin-TB (8 μg/well), C3-Biotin-TB (100 ng/well) AZGP1-Biotin-TB (200 ng/well) in competition with different concentration of native protein (HSA from 5 to 0.005 μg/well, C3 from 50 to 0.04 ng/well, AZGP1 from

50 to 0.04 ng/well) were added. The plate was covered and incubate for 60 min at room temperature on a plate shaker. Magnetic separation was performed for 30 sec and the plate was washed with 3 × 100 μl of PBS-BN. About 50 μl/well SAPE 4 μg/mL in PBS-BN was added. The plate was covered and incubate for 30 min at room temperature on a plate shaker. Magnetic separation was performed for 30 sec, then the plate was washed with 3 × 100 μl of PBS-BN. Microspheres were resuspended in 100 μl/well PBS-BN and analyzed 50 μl on the Luminex analyzer according to the system manual.

Statistical analysis. Descriptive analysis was made using median values and 95% Confidence Interval (CI). The differences were calculated using the Mann–Whitney's *U*-test for non parametric independent continuous variables. The BX plots described each variable (This represents 95 percentiles of all variables. Horizontal black bar in the grey boxes, the median value; bottom and top horizontal bars, minimum and maximum values). SPSS software, IL (version 17.00, SPSS, Chicago) was used for statistical analysis. A P value of less than 0.05 was considered to indicate statistical significance.

Results

Evaluation of a binding antibodies protocol

The accomplished tests indicated that the antibodies bind to the carboxylated beads of the Luminex for AZGP1 and HAS. A different strategy, based on indirect binding via Protein G (protein binding the constant fraction of the IgG) was successfully used for complement C3. The general evaluation of the various coupling strategies, lead to the optimization of the two procedures of different binding for the three proteins. The strategies are summarized in Table 2 and described in detail in the method section.

TABLE 2. The two procedures of different binding for the three proteins

Protein	Strategy
AZGP1	Direct binding with the antibody with COOH–beads
HSA	Direct binding with the antibody with COOH–beads
C3	Antibody binding mediated by Protein G

Development of a Luminex test prototype for the quantification of C3, albumin, and AZGP1 in serum

For the development of the multiplex test, it was assessed whether it was possible to create a mix of single standard for the three proteins. For this reason, a three-plex test was carried out. The test indicated the possibility to create a calibration three-plex curve. As illustrated in Figure 1, the three proteins were correctly calibrated in three-plex. The following step was to look for a single solution for the development of the three-plex. For this reason, it was verified whether the conditions of the C3 and HSA were applicable to the AZGPI and vice versa, following the procedure described in the method section. It is shown that the 1:1000 dilution is not applicable to AZGP1, as there is no reduction of the intensity of the fluorescence, a sign that a competition occurred between the biotinylated protein and the serum protein (data not shown). Similarly, the 1:4 dilution of the serum cannot be applied to the C3 protein and the HSA, as the high concentration of the protein in the serum, nullifies the fluorescence signal (data not shown). Therefore, also in this case, it was impossible to assess the protein concentration in the biological sample.

For this reason a 2-plex HSA-C3 + 1-plex AZGP1 test was set up. This test was carried out following the procedure described in the method section. In order to guarantee the rationality of the accomplished test, some recovery preliminary tests were done, by adding to the serum known concentrations of non-biotinylated protein. The test was carried out following the procedure described in the method section. Table 3 shows the results of this test: a rational decrease of the signal, adding a known quantity of biotinylated protein to the serum was seen.

Quantification of C3, HSA, and AZGP1 in the serum of endometriosis patients using Luminex test prototype

In order to definitively confirm the effectiveness of the Luminex test prototype, we decided to assess the protein levels of C3, HAS, and AZGP1 in a small cohort of endometriosis patients and healthy controls, selected as described in the method section. In Figure 2, the results obtained with this test are depicted. Interestingly, the patients missed with one marker, where picked up with the two others. In Figure 3 a BX plots described each variable for AZGP1 (Part A) and HAS (Part B). Statistical analysis by the Mann–Whitney's *U*-test for non-parametric independent continuous variables confirmed the significance of the different expressions for these two proteins ($P = 0.06$ for AZGP1 and $P = 0.01$ for HAS).

Discussion

The possibility to perform an early definitive diagnosis of endometriosis, without recurring to surgery, would allow to identify endometriosis patients in a short window of time. The

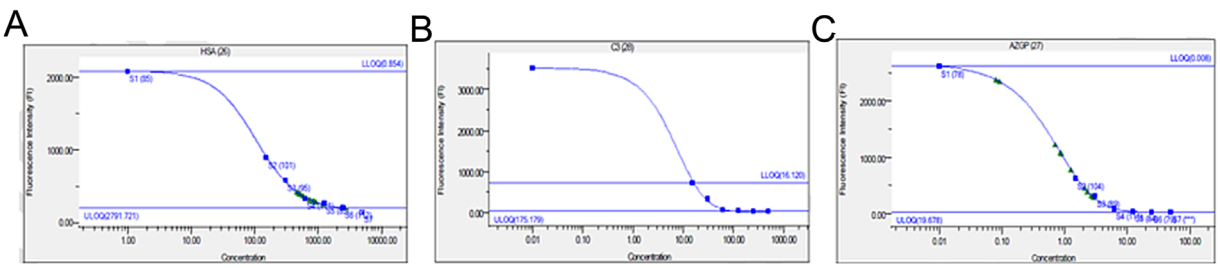

Fig. 1. Calibration three-plex curves for (A) HAS, (B) C3, and (C) AZGP1.

TABLE 3. Recovery preliminary tests

Input	FI
Standard	588
Standard + serum	437
Standard + serum + 0.1 μg HAS	380
Standard	271
Standard + serum	264
Standard + serum + 0.01 μg C3	18
Standard	466
Standard + serum	407
Standard + serum + 0.01 μg AZGP1	307

FI, fluorescence intensity.

clinical impact is substantial: patients would avoid a significant number of usefulness diagnostic procedures and the interval time between unset of the symptoms and definitive diagnosis of endometriosis would be greatly shortened (Bulun, 2009).

Indeed, several papers have proposed potential diagnostic biomarkers markers detected in the peritoneal fluid and serum, but up to now, none of these markers has proven to be completely reliable to permit its use alone for diagnosis (Seeber et al., 2008).

Our research group has recently identified by the 2DE-gel approach, three different potential bio-markers (HAS, AZGP1, and complement C3), whose expression was significantly different in endometriosis patients respect to healthy controls (Signorile and Baldi, 2014, 2015c). This preliminary observation on a small number of patients was confirmed on a larger cohort of endometriosis patients with slightly different results for the three markers (Signorile and Baldi, 2014, 2015c). We noticed that diagnostic potential of the three markers was not completely overlapping and that patients missed by one of the markers were identified by the other two markers. Therefore, it is reasonable to think that the determination of these three markers would achieve a diagnostic value. Leaving from this observation, we decided to set up the experimental condition to analyze simultaneously the three markers in the serum of the patients, by taking advantage of the Luminex technology. Indeed, in this article we have precisely defined the technical characteristics of this new assay.

In detail, for the development of a prototype multiplex assay for the simultaneous determination and the quantification of the three proteins, HSA, complement C3, and AZGP1, we have carried out the following activities: evaluation of a binding antibodies protocol, evaluation of the feasibility of a protocol of a single Luminex competitive assay, evaluation of the feasibility of a protocol of a multiplex Luminex competitive assay. As a binding antibodies protocol, a strategy that provides the binding to the magnetic carboxylated beads of the Luminex through the amino groups present on the molecules, was used. This strategy is the one mainly used for the Luminex technology. To avoid the alteration of the structure of the antibodies that are stabilized by disulfide bridges, the strategy that provides the binding of the antibodies to the beads through the cysteine residues, was not considered. Moreover, the method that provides the conjugation of streptavidin to the beads and the successive binding of biotinylated antibodies, was not considered, because this protocol requires high costs. The accomplished tests indicated that the antibodies bind to the carboxylated beads of the Luminex for AZGP1 and HAS. A different strategy, based on indirect binding via Protein G (protein binding the constant fraction of the IgG), was successfully used for complement C3. Concerning the valuation of the feasibility of a protocol of a single Luminex competitive assay, the antibody conjugated to the beads was left to incubate with the serum in the presence of the biotinylated protein. The competition between the biotinylated protein and the protein in the serum provided a quantitative determination of the latter. For the development of this type of test, a titration curve in known constant

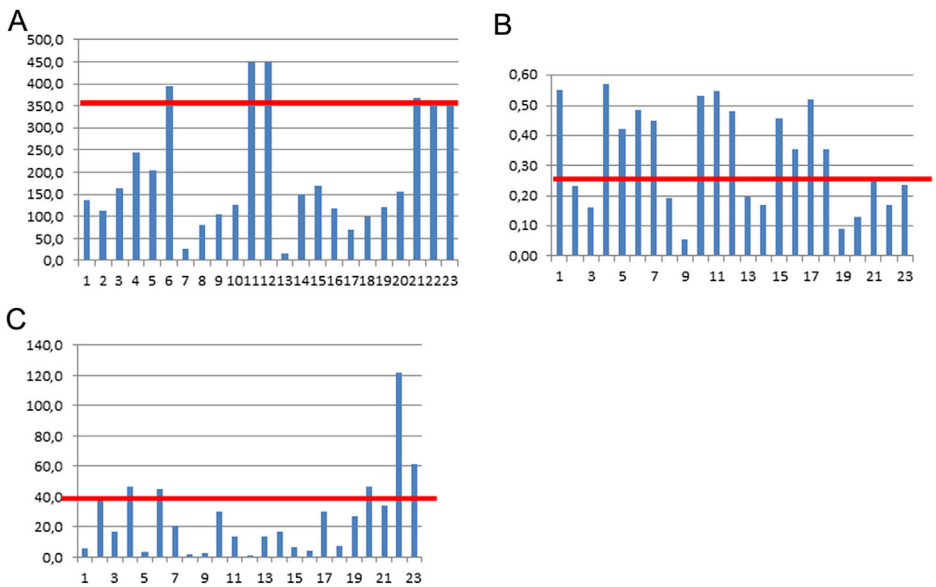

Fig. 2. A) Values for AZGP1 in 20 endometriosis patients and three healthy controls; the red line indicates the reported normal values for AZGP1 in healthy individuals. B) Values for C3 in 20 endometriosis patients and three healthy controls; the red line indicates the reported normal values for C3 in healthy individuals. C) Values for HSA in 20 endometriosis patients and three healthy controls; the red line indicates the reported normal values for HSA in healthy individuals.

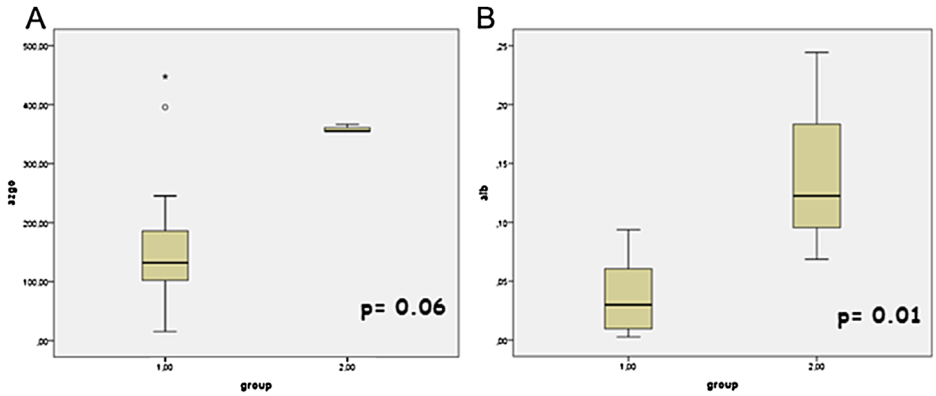

Fig. 3. BX plots described each variable for AZGP1 (Part A) and HAS (Part B). This represents 95 percentiles of all variables. Horizontal black bar in the gray boxes, the median value; bottom and top horizontal bars, minimum and maximum values.

concentration of the biotinylated protein in competition with known increasing concentrations of non-biotinylated protein, was initially realized. To develop this protocol, we have also optimized a procedure of biotinylation of the three proteins, as the C3 and AZGP1 in their biotinylated form were not commercially available. Concerning, the evaluation of the feasibility of a protocol of a multiplex Luminex competitive assay, we assessed if it was possible to quantify the three proteins in the same test. First it was evaluated whether it was possible to make a calibration curve of the three proteins together. Due to the difference of concentration between HAS and C3 (expressed in mg/ml) and AZGP1 (expressed in ng/ml), that resulted also from the protocols in single, the greater difficulty was to find a dilution of a biological sample optimal for the three-plex. Indeed, the difference of the concentration of the three proteins did not allow to define a single serum dilution which permitted the simultaneous determination of the same, therefore the best solution for the test was a 2-plex (HSA + C3) + 1-plex (AZGP1). Even if there are no problems to create a three-plex calibration curve, the production of a 2-plex +1-plex curve is, however, recommended in order to create a product which presents itself in the same format for the determination of calibrators and samples.

Finally, we have also confirmed the diagnostic potential of the three markers with a pivotal study on 20 endometriosis patients. The cohort included only three controls, but this did not diminished the value of the observation, as the normal expression levels of these three proteins in healthy individual is well characterized (35 mg/L for Zn-alpha2-glycoprotein, 40 mg/ml for albumin and 0.25 mg/ml for complement C3 precursor) and, using these levels as cut-off, the diagnostic value of the biomarkers was statistically confirmed (Stejskal et al., 2008; Signorile and Baldi, 2014, 2015c).

In conclusion, the data of our study demonstrate for the first time the possibility to use a multiplex assay based on Luminex technology to individuate endometriosis patients with a fast and non-invasive test by means of routinely used biochemical techniques. The potential clinical implications are relevant, especially if we consider women of reproductive age. In fact, an effective non-invasive diagnostic test for endometriosis would allow to select these patients for laparoscopic treatment of endometriosis very early and this has been proven to improve fertility (Kennedy et al., 2005); furthermore, it may avoid progression of the disease. We have also shown in our previous works, that this differentially expression is not influenced by the cycle phase and it is independent from endometriosis stage, thus proving that the hormonal input and the effects of the disease on the normal physiology of the body do not have any influence on the diagnostic performance (Signorile and Baldi, 2014, 2015c).

Further studies on large cohorts of endometriosis patients performed in different institutions are required to confirm the diagnostic value of this assay and to propose it to the scientific community has an effective diagnostic tool for endometriosis.

Acknowledgment

PGS and AB are authors of a patent application (WO 2013/171655) related to the themes of the article.

Literature Cited

Bai X, Liu Z, Gottschalk M, Zheng H, Xu J. 2015. Simultaneous detection of 33 streptococcus suis serotypes using the luminex xTAG(r) assay. J Microbiol Method 117:95–99.
Baldi A, Campioni M, Signorile PG. 2008. Endometriosis: Pathogenesis, diagnosis, therapy and association with cancer. Oncol Rep 19:843–846.
Ballard KD, Lowton K, Wright JT. 2006. What's the delay? A qualitative study of women's experience of reaching a diagnosis of endometriosis. Fertil Steril 85:1296–1301.
Benagiano G, Brosens I. 2006. History of adenomyosis. Best Pract Res Clin Obstet Gynaecol 20:449–463.
Bulun SE. 2009. Endometriosis. N Engl J Med 360:268–279.
Crispi S, Piccolo MT, D'Avino A, Donizetti A, Viceconte R, Spyrou M, Calogero RA, Baldi A, Signorile PG. 2013. Transcriptional profiling of endometriosis tissues identifies genes related to organogenesis defects. J Cell Physiol 228:1927–1934.
D'Hooghe TM, Mihalyi AM, Sisma P, Kyama CK, Peeraer K, De Loecker P, Meeuwis L, Segal L, Meuleman C. 2006. Why we need a noninvasive diagnostic test for minimal to mild endometriosis with high sensitivity. Gynecol Obstet Invest 62:136–138.
Dunbar SA. 2006. Applications of luminex xmap technology for rapid, high-throughput multiplexed nucleic acid detection. Int J Clin Chem 363:71–82.
Fassbender A, Waelkens E, Verbeeck N, Kyama CM, Bokor A, Vodolazkaia A, Van de Plas R, Meuleman C, Peeraer K, Tomassetti C, Gevaert O, Ojeda F, De Moor B, D'Hooghe T. 2012. Proteomic analysis of plasma for early diagnosis of endometriosis. Obstet Gynecol 119:276–285.
Fuldeore M, Chwalisz K, Marx S, Wu N, Boulanger L, Ma L, Lamothe K. 2011. Surgical procedures and their cost estimates among women with newly diagnosed endometriosis: A US database study. J Med Econ 14:115–123.
Gajbhiye R, Sonawani A, Khan S, Suryawanshi A, Kadam S, Warty N, Raut V, Khole V. 2012. Identification and validation of novel serum markers for early diagnosis of endometriosis. Hum Reprod 27:408–417.
Giudice LC, Kao LC. 2004. Endometriosis. Lancet 364:1789–1799.
Hadfield R, Mardon H, Barlow D, Kennedy S. 1996. Delay in the diagnosis of endometriosis: A survey of women from the USA and the UK. Hum Reprod 11:878–880.
Kennedy S, Bergqvist A, Chapron C, D'Hooghe T, Dunselman G, Greb R, Hummelshoj L, Prentice A, Saridogan E. 2005. ESHRE guideline for the diagnosis and treatment of endometriosis. Hum Reprod 20:2698–2704.
Lynch HE, Sanchez AM, D'Souza MP, Rountree W, Denny TN, Kalos M, Sempowski GD. 2014. Development and implementation of a proficiency testing program for luminex bead-based cytokine assays. I Immunol Meth 409:62–71.
May KE, Conduit-Hulbert SA, Villar J, Kirtley S, Kennedy SH, Becker CM. 2010. Peripheral biomarkers of endometriosis: A systematic review. Hum Reprod 16:651–674.
Mihalyi A, Gevaert O, Kyama CM, Simsa P, Pochet N, De Smet F, De Moor B, Meuleman C, Billen J, Blanckaert N, Vodolazkaia A, Fulop V, D'Hooghe TM. 2010. Non-invasive diagnosis of endometriosis based on a combined analysis of six plasma biomarkers. Hum Reprod 25:654–664.

Revised American Society for Reproductive Medicine classification of endometriosis. 1997. Fertil Steril 67:817–821.
Seeber B, Sammel MD, Fan X, Gerton GL, Shaunik A, Chittams J, Barnhart KT. 2008. Panel of markers can accurately predict endometriosis in a subset of patients. Fertil Steril 89:1073–1081.
Signorile PG, Campioni M, Vincenzi B, D'Avino A, Baldi A. 2009a. Rectovaginal septum endometriosis: An immunohistochemical analysis of 62 cases. In Vivo 23:459–464.
Signorile PG, Baldi F, Bussani R, D'Armiento MR, De Falco M, Baldi A. 2009b. Ectopic endometrium in human fetuses is a common event and sustains the theory of mullerianosis in the pathogenesis of endometriosis, a disease that predisposes to cancer. J Exp Clin Cancer Res 9:28–49.
Signorile PG, Baldi A. 2010a. Endometriosis: New concepts in the pathogenesis. Int J Biochem Cell Biol 42:778–780.
Signorile PG, Spugnini EP, Mita L, Mellone P, D'Avino A, Bianco M, Diano N, Caputo L, Rea F, Viceconte R, Portaccio M, Viggiano E, Citro G, Pierantoni R, Sica V, Vincenzi B, Mita DG, Baldi F, Baldi A. 2010b. Pre-natal exposure of mice to bisphenol A elicits an endometriosis-like phenotype in female offspring. Gen Comp Endocrinol 168:318–325.
Signorile PG, Baldi F, Bussani R, D'Armiento M, De Falco M, Boccellino M, Quagliuolo L, Baldi A. 2010c. New evidence of the presence of endometriosis in the human fetus. Reprod Biomed Online 21:142–147.
Signorile PG, Baldi F, Bussani R, Viceconte R, Bulzomi P, D'Armiento M, D'Avino A, Baldi A. 2012. Embryologic origin of endometriosis: Analysis of 101 human female fetuses. J Cell Physiol 227:1653–1656.
Signorile PG, Baldi A. 2014. Serum biomarker for the diagnosis of endometriosis. J Cell Physiol 229:1731–1735.
Signorile PG, Baldi A. 2015a. New evidence in endometriosis. Int J Biochem Cell Biol 60:19–22.
Signorile PG, BAldi A. 2015b. A tissue specific magnetic resonance contrast agent, Gd-AMH, for diagnosis of stromal endometriosis lesions: A phase I study. J Cell Physiol 230:1270–1275.
Signorile PG, Baldi A. 2015c. Supporting evidences for potential biomarkers of endometriosis detected in peripheral blood. Data Brief 5:971–974.
Stejskal D, Karpísek M, Reutová H, Stejskal P, Kotolová H, Kollár P. 2008. Determination of serum zinc-alpha-2-glycoprotein in patients with metabolic syndrome by a new ELISA. Clin Biochem 41:313–316.