Prototype Salivary Assay for Quantification of Two Biomarkers for *In Vitro* Diagnosis of Endometriosis

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ABSTRACT: Endometriosis, a very common disease in women, is characterized by endometrial structures outside the uterine cavity. The lack of a reliable noninvasive diagnostic test and the often nonspecific symptoms of this pathology are responsible for the delay in definitive diagnosis of this disease. Recently, through a proteomics approach, our research group has identified two potential diagnostic markers for endometriosis in serum (Zn-alpha2-glycoprotein and complement C3 precursor). In this article, we describe the experimental conditions of a simple ELISA for rapid quantification of these two biomarkers in the saliva of patients with endometriosis. Finally, preliminary experiments on a small cohort of patients and controls have confirmed the potential diagnostic value of this assay.

KEY WORDS: biomarker, ELISA, saliva, Zn-alpha2-glycoprotein, complement C3

I. INTRODUCTION

Endometriosis is a very common gynaecological disease, whose principal characteristic is the presence of endometrium-like tissue outside the uterine cavity.[1] It is reported in up to 10% of reproductive-aged women.[2] The most recognized locations of endometriosis lesions are the pelvic peritoneum and ovaries, but recent data from our research group have shown that the highest incidence is indeed under the peritoneal surface and in the pouch of Douglas.[3] Interestingly, this form of endometriosis, defined as deep-penetrating endometriosis, is associated with more marked symptoms.[4]

Despite the fact that this clinical entity causes striking morbidity and infertility, and requires extensive medical and surgical treatments associated with noteworthy costs and risks,[5] endometriosis is still a mysterious disease. In fact, its pathogenesis, diagnosis, and therapy are not yet completely defined. Concerning pathogenesis, the current dispute is about the time and mechanism through which endometriosis implants are formed. Until today, the most acknowledged pathogenetic theory is the one anticipated a century ago.[6] It claims that ectopic implants of menstrual shedding can arrive at the abdominal cavity through the fallopian tubes and grow on the peritoneal surface. Other hypothesized pathogenetic mechanisms are the celomic metaplasia model and the endometrial stem cell recruitment model.[7,8] Recently, various research groups have generated considerable experimental data supporting a different theory based on the persistence of remnants of the embryonic Müllerian ducts in ectopic locations and its correlation with the endometriosis condition.[9–12] Molecular evidence points the ectopic dislocation of these embryonic remnants toward perturbation in the fine-tuning of female genital development during a critical window of time in fetal life; these implants keep silent until puberty when, stimulated by hormonal input, they rise into endometriosis lesions.[13,14]

As a matter of fact, despite its high prevalence, endometriosis is still insufficiently identified and diagnosis time ranges from 4 to 11 years, with 65% of women being at first misdiagnosed.[15] Until now, none of the many biomarkers in peripheral blood proposed for endometriosis have been definitely validated.[16,17] As a logical consequence, laparoscopy in association with histological examination is still the diagnostic gold standard. However, there are significant limitations for laparoscopy. Among others, they are the invasiveness

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of the surgical procedure[18] the observation that operative laparoscopy is performed in a significant number of patients that are disease-free,[19] and the dependence of the consistency of diagnosis on the experience of the medical professional performing the laparoscopy.[20]

It is obvious that characterization of reliable *in vitro* diagnostic tests for the noninvasive diagnosis of endometriosis would be of great importance in clinical practice, reducing the time of diagnosis and allowing early identification of patients suitable for surgical treatment, which has been reported to decrease pain and increase fertility.[21]

Using a proteomic approach based on 2DE-gel analysis, we have identified three potential biomarkers for the *in vitro* diagnosis of endometriosis: Zn-alpha2-glycoprotein (AZGP1), human serum albumin (HSA), and complement C3 precursor (C3).[22–24] Interestingly, via ELISA of blood serum, we have determined a statistically significant diagnostic value for these biomarkers.[22–24]

In this study, we developed a noninvasive salivary test for the evaluation of AZGP1 and C3, and investigated its potential diagnostic value among a small cohort of endometriosis patients.

II. MATERIALS AND METHODS

A. Study Population

Saliva samples from 16 endometriosis patients and 9 healthy controls were recruited by the Centro Italiano Endometriosi in Rome. The study was approved by the ethical committee of the Fondazione Italiana Endometriosi. All subjects signed a written informed consent. The diagnosis of endometriosis was confirmed by laparoscopy and histology. Five subjects in the control group were women of reproductive age regularly cycling and spontaneously ovulating and without menstrual pain. Four subjects were healthy adult males.

B. Recombinant Proteins and Antibodies

AZGP1 and C3 His-tag proteins were expressed in mammalian cells (HEK 293-F; Thermo Fisher Scientific, Waltham, MA) and then purified with immobilized metal affinity chromatography (IMAC) (Merk Life Science Srl, Milan, Italy). Polyclonal antibodies were developed after rabbit immunization with the AZGP1 and C3 recombinant antigens. The specific IgG antibodies from immune sera were purified by Protein A affinity chromatography from specific immune sera.

In a second step, a few aliquots of the same polyclonal antibodies were conjugated with NHS-Biotin for detection of streptavidin HRP immune complexes.

C. Sample Collection

Saliva samples were collected in sputum collection containers (Kartell, Noviglio, Italy). After rinsing the mouth three times with water for three seconds, the sputum was collected and stored immediately at −20°C before testing.

D. Test Setup

For each protein, ELISA was performed on 96-well plates with high binding capacity. The plates were coated with 100 µL/well of specific polyclonal antibody diluted 10 µg/mL in physiological conditions. They were then incubated overnight at 4°C. After four washes with phosphate buffer saline (PBS) with added Tween 20 at 0.05% v/v, the plates were blocked for one hour at 37°C and then washed again. Samples were diluted 1:100 v/v and 1:50 v/v for AZGP and C3 respectively in modified RIPA buffer (Merk Life Science) and subsequently, they were dispensed in volumes of 100 µL per well. Standard curve dilutions were prepared with purified recombinant proteins C3 and AZGP1 diluted in RIPA buffer and put in triplicate wells for each concentration. The plates were incubated for one hour at 37°C and then washed prior to dispensing 1 µg/mL diluted biotinylated antibodies in PBS with BSA w/v added. In the next step the streptavidin HRP was added at 100 µL for well-diluted 1:100 v/v in PBS and BSA 1% w/v, and maintained at 37°C for one hour. After further washing, the immune complexes were determined by addition of a chromogenic substrate, ABTS, and then the colored reaction was read after 20 minutes with a microplate reader at 405

nm. Biomarker concentrations were determined on a standard curve with known concentrations of AZGP1 and C3, and calculated by linear regression in Microsoft Excel.

E. Statistical Analysis

All statistical analyses were performed using a Student's *t*-test in Microsoft Excel. The difference between samples was considered significant at $p < 0.05$.

III. RESULTS

A. Experimental Conditions for Standardization of a Salivary Test Protocol

The methodology was optimized by important modifications for standardization and test preparation in an industrialization phase, making it suitable for routine production. First, a coating pad was devised to make plate sensitization stable over time. Three buffers were tested to identify the best one in terms of performance and stability: PBS, pH 7.4; 20-mM TRIS/HCl, pH8; and 50-mM carbonate buffer, pH9.6. After several tests, the physiological buffer PBS was chosen. The ideal concentration of the capture antibody in the optimal coating buffer was studied at 1 to 10 µg/mL. The saturating concentration was found to be 10 µg/mL. For determining the best signal, two types of streptavidin HRP (horseradish peroxidase) together with two chromogenic substrates were evaluated (TMB and ABTS). Based on these experiments, a streptavidin with poly HRP associated with the ABTS substrate was chosen. Furthermore, the plate-staining time was defined to highlight the immune complexes and to eliminate any type of background. Reading of the colored plates was performed after 10, 20, 30 and 45 minutes, with the best setting time determined to be 20 minutes. In addition, dilution concentrations of saliva samples were defined to determine the signal without false or undetectable signals. For this purpose, saliva samples were tested at three dilutions (1:50 to 1:200). The optimal dilutions for C3 and AZGP1 were determined to be 1:50 v/v and 1:200 v/v, respectively.

B. Development of a Salivary Test Prototype for Quantification of C3 and AZGP1

Using the method reported in "Materials and Methods," reproducibility tests were carried out on the calibration lines diluted in buffer for standardization of the methodology. Calibration lines were developed on eight concentrations in serial 1:2 dilution of the recombinant protein, starting at 30 ng/mL. Once the method had been verified, both negative and positive saliva samples of patients with a certain diagnosis were tested as described in "Materials and Methods." Figure 1 shows the calibration lines determined via linear regression analysis and the related equation for both AZGP and C3.

C. Quantification of C3 and AZGP1 in the Saliva of Endometriosis Patients Using the Salivary Test Prototype

The reproducible methodology allowed us to develop a prototype with which saliva with a known diagnosis was evaluated and the proteins present on the calibration line were quantified. As shown in Figs. 2 and 3, we found a clear difference between the negative and positive samples for both markers, despite the small number of samples.

Figure 4 shows median values for AZGP1 and C3 plotted in histograms. The difference between the two cohorts was significant for both proteins (p 0.05). Both antigens showed a significant increase in patients compared with controls.

IV. DISCUSSION

Early conclusive diagnosis of endometriosis by means of a noninvasive test would allow clinicians to recognize endometriosis in a short time window, avoiding time-consuming and invasive procedures. The clinical impact is noteworthy in that patients would be spared useless diagnostic procedures; also, the time between commencement of symptoms and final diagnosis would be significantly abbreviated.

Indeed, several papers have proposed potential diagnostic biomarkers detected in the peritoneal fluid and serum, but achieved no definitive results.[17]

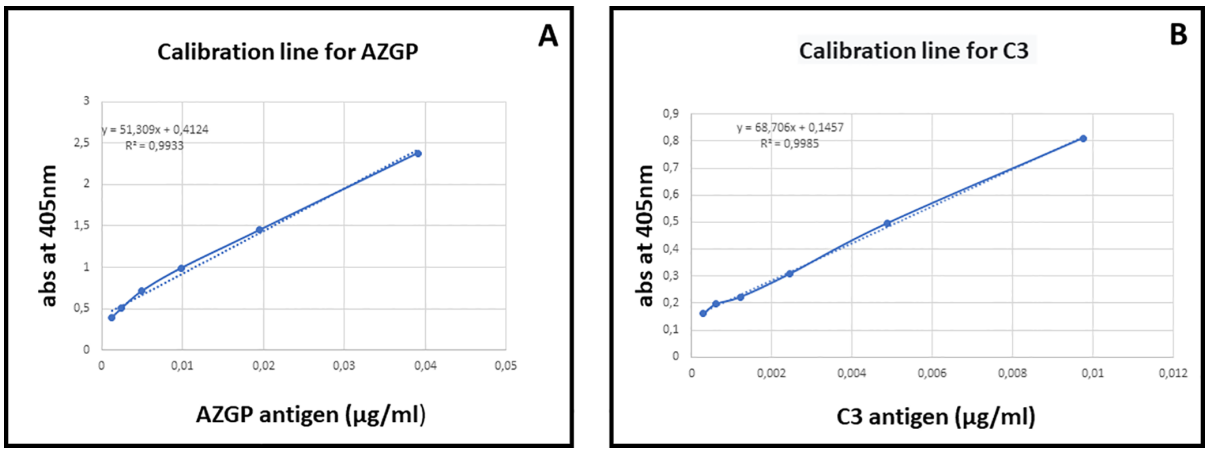

FIG. 1: Calibration curves

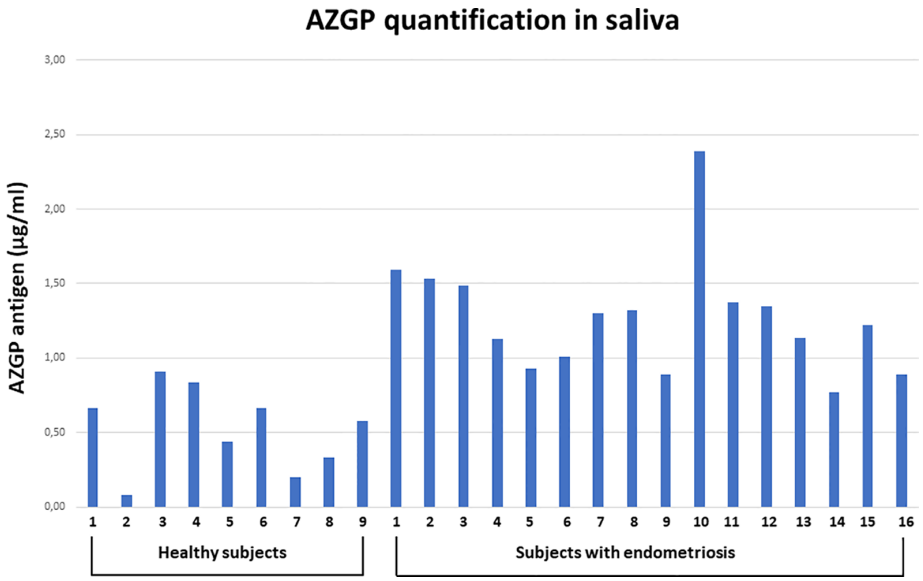

FIG. 2: AZGP1 values from 16 endometriosis patients and 9 controls

Recently, a molecular bioinformatics approach based on microRNA-sequencing analysis was proposed as a way to build a saliva signature of endometriosis.[25]

Using a proteomic approach, our research group recognized two biomarkers, AZGP1 and C3, whose expression levels were significantly dissimilar in the serum of endometriosis patients and that of controls.[22–24] Based on this preliminary observation, we defined experimental conditions under which we could analyze these biomarkers in saliva using ELISA. In our previous work, we defined a third marker (albumin), which we have so far been unable to reliably detect in saliva. The decision to use saliva in our quantitative analyses was based on the simple observation that sampling would be much less invasive than sampling serum and would make viable an economical and easy-to-use diagnostic test for a large number of patients. This is very important in view of the high incidence of endometriosis.[3,4] In this study, we precisely defined this new assay.

The new quantitative assay, based on ELISA, detects new biomarkers from saliva that are related

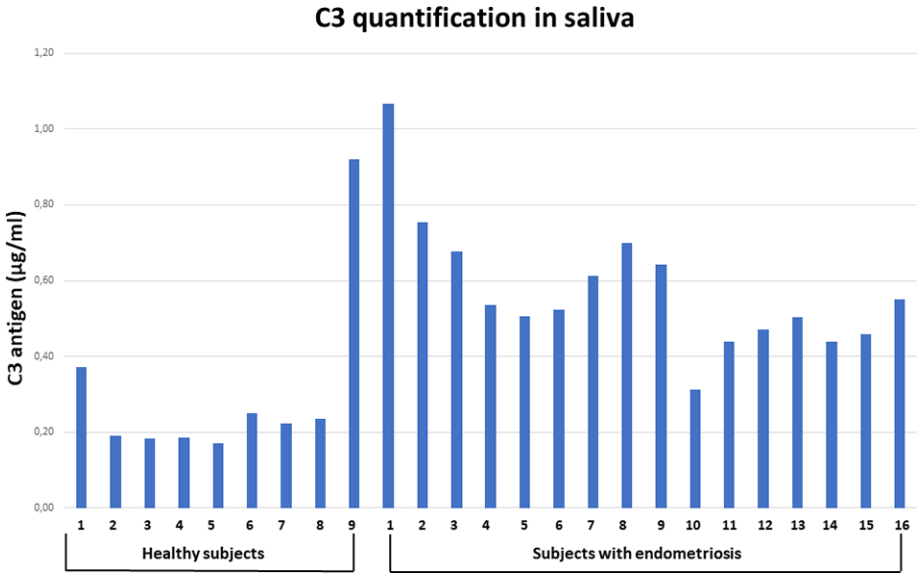

FIG. 3: C3 values from 16 endometriosis patients and 9 controls

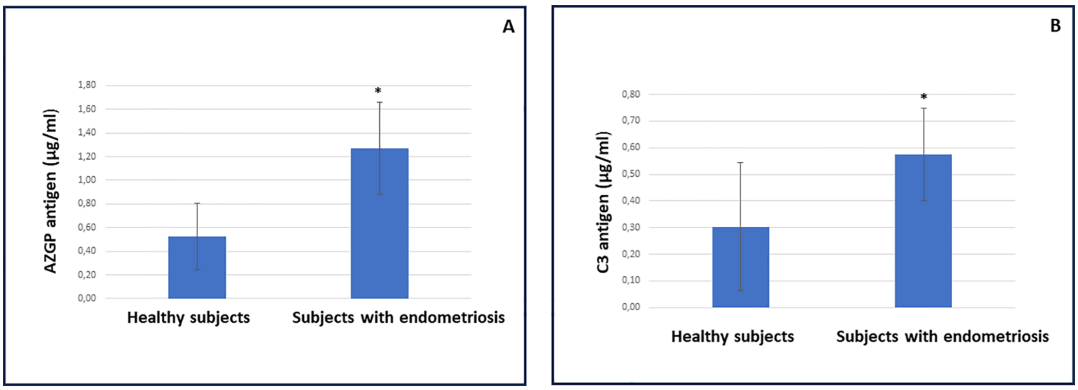

FIG. 4: Median value for AZGP1 (A) and C3 (B) in controls and endometriosis patients, indicating standard deviation; * = significant statistical value of patients compared with controls

to endometriosis pathology. It provides a diagnostic parameter in a short time without invasive procedures. Moreover, it is reproducible and is based on a technique widely used by laboratory personnel. The methodology can be carried out on many samples by determining the concentration of the two markers simultaneously.

Finally, we established the diagnostic potential of AZGP1 and C3 expression levels in saliva via noninvasive saliva collection and analysis of their expression compared with that in healthy controls. Despite the small number of patients and controls, the results showed statistical significance for both biomarkers. Nevertheless, pathological levels will need to be validated by expanding the study of these biomarkers to include adequately large patient cohorts.

A potential salivary test for endometriosis based on microRNA-sequencing analysis was recently reported.[25] The test described in our work differs from a molecular test in that it is much simpler and cheaper, and thus suitable for large-scale use. Our test is an ideal candidate for the diagnosis of endometriosis in large numbers of patients. The clinical

implications are relevant, especially if we consider women in their reproductive years. In fact, quickly identifying women with endometriosis would allow immediate laparoscopic treatment to potentially increase fertility.[26] In any case, early diagnosis would prevent disease progression.

It will be essential to enroll significantly greater numbers of patients and controls in different institutions to define with certainty the diagnostic value of AZGP1 and C3 expression in saliva and to propose it to the scientific community as a diagnostic test for endometriosis.

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AUTHOR CONTRIBUTIONS

PGS and AB were responsible for conceptualization, formal analysis, writing, and original draft preparation; SD and RC were responsible for methodology and statistical analysis; PGS was responsible for funding acquisition.

CONFLICT OF INTEREST

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

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