Endometriosis pathogenesis: the relationship between oxidative stress, fibrosis and immunological dysfunction

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UNIVERSITAT DE BARCELONA

DOCTORAL THESIS

Programa de Doctorat en Medicina

Universitat de Barcelona

ENDOMETRIOSIS PATHOGENESIS: THE RELATIONSHIP BETWEEN OXIDATIVE STRESS, FIBROSIS AND IMMUNOLOGICAL DYSFUNCTION

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A thesis submitted by Iñaki González Foruria for the PhD degree (Doctor in Medicine, Faculty of Medicine, University of Barcelona) including the mention of "International Doctor" under the direction of Francisco Carmona Herrera, Professor of Obstetrics and Gynaecology at Barcelona University and Charles Chapron, Professor of Obstetrics and Gynaecology at University Paris Descartes, Faculty of Medicine, Paris, France.

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We confirm that Iñaki González Foruria has conducted under our supervision the studies presented in the thesis "Endometriosis pathogenesis: the relationship between oxidative stress, fibrosis and immunological dysfunction". The present thesis has been structured following the normative for PhD theses as a compendium of publications for the degree of International Doctor in medicine, and that the mentioned studies are ready to be presented to the Tribunal.

Francisco Carmona Herrera Charles Chapron

Barcelona, October 2017

PRESENTATION

This thesis project has been structured following the normative for PhD thesis as a compendium of publications. The studies included in the thesis belong to the same research line leading to two articles already published in international journals:

Article 1

Dysregulation of the ADAM/Notch signalling pathway in endometriosis: from oxidative stress to fibrosis.

González-Foruria I, Santulli P, Chouzenoux S, Carmona F, Chapron C, Batteux F. Molecular Human Reproduction. 2017 May; 9:1-12.

State: published

Impact factor: 3.585

Quartile: 1st

Article 2

Soluble ligands for the NKG2D receptor are released during endometriosis and correlate with disease severity.

González-Foruria I, Santulli P, Chouzenoux S, Carmona F, Batteux F, Chapron C.

PLOS ONE. 2015; 10(3): e0119961

State: published

Impact factor: 4.411

Quartile: 1st

GRANTS AND FINANCIAL SUPPORT

Iñaki González Foruria was awarded with a grant from Fundación Alfonso Martín Escudero in order to develop the project of this thesis in the Department of Obstetrics and Gynaecology II and Reproductive Medicine at Cochin University Hospital, University Paris Descartes, Faculty of Medicine, Paris, France.

These works have also been supported by grants from University Paris Descartes and INSERM.

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ABBREVIATIONS

α-SMA: Alpha-Smooth Muscle Actin

ADAM: A Disintegrin and Metalloproteinases

AOPP: Advanced Oxidation Protein Products

BMI: Body Mass Index

Cs: Eutopic Endometrial Stromal Cells from controls

DIE: Deep Infiltrating Endometriosis

ELISA: Enzyme-Linked Immunosorbent Assay

EMT: Epithelial-Mesenchymal Transition

Es: Eutopic Endometrial Stromal Cells from DIE patients

FMT: Fibroblast-to-Myofibroblast Transdifferentiation

I-CAM: Intercellular Adhesion Molecule

MHC: Major Histocompatibility Complex

MIC: MHC class-I Chain related

MMPs: Matrix Metalloproteinases

NCPP: Non-Cyclic Chronic Pelvic Pain

NICD: Notch Intracellular Domain

NK: Natural Killer

NKG2D: Natural Killer Group 2, member D

NKG2DL: Natural Killer Group 2, member D Ligands

OC: oral contraceptives

OMA: Ovarian Endometrioma

PF: Peritoneal Fluid

Ps: Eutopic Endometrial Stromal Cells from DIE patients

rAFS: revised American Fertility Society

ROS: Reactive Oxygen Species

SMM: Smooth Muscle Metaplasia

SUP: Superficial Peritoneal Endometriosis

TACE: Tumor Necrosis Factor-Alpha Converting Enzyme

TNF: Tumor Necrosis Factor

ULBP: UL16 Binding Protein

1. INTRODUCTION

1.1. Endometriosis: epidemiology, clinical forms and pathophysiological theories

Endometriosis is a benign gynaecological disease characterized by the presence of endometrial tissue outside the uterine cavity, primarily on the pelvic peritoneum and ovaries (1). The most common clinical features of endometriosis are dysmenorrhea, chronic pelvic pain, pain during intercourse, and infertility. However, the clinical symptoms do not always correlate with the extent of the disease (2), and for this reason, the diagnosis of the disease is often delayed between 7-10 years (3, 4).

The real prevalence of endometriosis is unknown. There are no published studies on representative samples of the general population. In general, it is difficult to compare estimates of prevalence because the previously published studies include women with different conditions, and are conducted in centres that apply different diagnostic criteria and exhibit different levels of clinical interest in endometriosis. However, and according to prevalence estimates, it is accepted that this inflammatory disorder may affect up to 10-15% of women of reproductive age, representing a major health issue (5, 6).

Depending on the extension and localization of the disease, three clinically distinct forms of endometriosis can be distinguished: endometriotic implants on the surface of the pelvic peritoneum (superficial peritoneal endometriosis -SUP-), ovarian cysts lined by endometrial mucosa (ovarian endometriomas -OMA-), and complex solid nodules of endometriotic tissue blended with fibromuscular tissue infiltrating deep in the peritoneum (deep infiltrating endometriosis -DIE-) (7, 8).

Each of these lesion types may be presented isolated or they may coexist at the same time (9).

There is no general agreement in the pathologic origin of endometriosis. Almost a century ago, Sampson proposed that small fragments of endometrial debris reached the peritoneal cavity through retrograde menstruation, implanting and proliferating on peritoneal surface or pelvic organs (10). In contrast, the coelomic metaplasia theory postulates that endometriosis originates from the metaplasia of cells lining the peritoneal cavity. After different hormonal, environmental or other stimuli, these peritoneal lining cells may transform into endothelial cell types following metaplasia. The basis of this hypothesis lies in embryological studies that show that the peritoneum and endometrium share a common embryonic origin: the coelomic epithelium (11). Likewise, the embryonic Mullerian rests theory suggests that endometriotic lesions arise after a concrete hormonal/environmental stimulus over remaining cells from Mullerian duct migration during embryonic development (12). Another hypothesis proposes that menstrual cells from eutopic endometrium migrate to other sites through veins or lymphatic vessels (13). More recently, a new proposal suggests that stem cells from the endometrium or multipotent cells from bone marrow may reach the peritoneal cavity and differentiate into endometriotic tissue (14, 15). When progenitor cells from endometrium basalis are shed at the time of menstruation, they may implant and create ectopic endometrium locations (16).

Nowadays, the retrograde menstruation hypothesis, initially proposed by Sampson, is the most accepted theory, as it is both intuitively attractive and biologically plausible. In favour of this theory is the finding of menstrual endometrial debris in peritoneal fluid (PF) of up to 90% of healthy women (17).

Further support for this etiology is derived from studies in patients with obstructed or compromised outflow tracts, where the prevalence of endometriosis is higher than in healthy women (18). In the same line, experiments creating a iatrogenic obstruction of the outflow tract in a non-human primate model result in endometriotic lesions inside the peritoneal cavity (19). Besides, the anatomic distribution of endometriotic lesions also favours Sampson's theory, as implants are more often located in the posterior compartment of the pelvis and in the left hemipelvis (20, 21). This fact may be explained by the accumulation of regurgitated menstrual debris in the posterior part of the peritoneal cavity under the influence of gravity and because the sigmoid colon contributes to the stasis of PF in the left hemipelvis, thereby extending the interval for endometrial cells to implant.

Nevertheless, Sampson's retrograde menstruation hypothesis cannot account for the complete development of endometriotic implants. Actually, further steps are needed to explain how endometrial cells can escape from immune clearance, the increased attachment and invasion to peritoneal epithelium and the autogeneration of local neurovascularity and fibrosis.

1.2. Basic research in endometriosis pathogenesis: increased oxidative stress, inflammation, fibrosis and immunological dysfunction

Thousands of research articles have been published regarding endometriosis pathogenesis in the last decades. However, more than a century after the description of the disease by Von Rokitansky (22), there is still a poor understanding of this disorder's origin.

Like in many other inflammatory conditions, oxidative stress has been proposed as a potential factor involved in the pathogenesis of endometriosis (23, 24). Reactive oxygen species (ROS) are chemically reactive molecules naturally produced within the mitochondria during normal oxygen metabolism. When there is an imbalance of ROS and antioxidants towards the former, oxidative stress impacts damaging proteins, lipids and DNA structure. An excessive liberation of ROS not only produces cellular damage but may also alter cellular function by regulating protein activity and gene expression (25, 26). In a large prospective laboratory study by Santulli *et al*. (27), protein oxidative stress markers (thiols, advanced oxidation protein products -AOPP-, protein carbonyl, and nitrates/nitrites) were assessed in PF of women undergoing abdomino-pelvic surgery for endometriosis or other reasons (controls). The authors found a significant increase of PF AOPP and nitrates/nitrites in patients suffering from DIE compared to controls. However, the other forms of endometriosis (SUP and OMA) did not show a significant increase of oxidative stress markers compared to controls. Likewise, other authors have performed similar studies evaluating different biomarkers in patients with endometriosis and in endometriosis animal models. The conclusions always point out the role of oxidative stress in endometriosis (28-31).

Endometriosis is a complex chronic inflammatory condition that associates an overproduction of prostaglandins, metalloproteinases, cytokines and chemokines. Inflammation itself generates a self-supporting loop that sustains and increases the development of the disease (32). Abundant bulk of evidence shows that a large number of acute inflammatory molecules are increased in women with endometriosis, especially in those suffering from the most severe forms of the

disease (DIE) (33-36). Interleukin-1β, interleukin-6, tumor necrosis factor (TNF), monocyte chemoattractant protein 1, and RANTES are some of the cytokines/chemokines responsibles of the molecular, genetic and immunological pathway alterations that are found in endometriosis. Conversely, it has been shown that other anti-inflammatory molecules (such as interleukin-19 and interleukin-22, from the interleukin-10 family) are decreased in these patients (37). Although inflammation is a crucial step towards the maintenance and progression of endometriosis, the origin of these molecules is still unclear. It is still to be determined whether any endogenous condition of the women or a primary alteration on endometrial cells that reach the peritoneal cavity may generate the onset of inflammatory mediators secretion (38).

Fibrosis surrounding endometriotic lesions is another of the main features of endometriosis, mainly in DIE (39). During the development of deep nodules, it is common to observe an excess of fibrosis that may result in subsequent adhesion and anatomic distortion (40, 41). Such fibrosis is very likely to be in part responsible for endometriosis-associated infertility, pelvic pain and even for the resistance to pharmacological treatment in some cases (42). Despite extensive documentation for fibrosis in this disorder, the concrete underlying mechanisms of fibrogenesis still remain poorly understood. It is known that during the course of granulation tissue formation, myofibroblasts synthesize the connective tissue matrix and express an elevated proportion of myofibroblastic differentiation markers, such as alpha-smooth muscle actin (α-SMA), type-I collagen and fibronectin. α-SMA is considered the most reliable marker of myofibroblastic differentiation (43), and α-SMA-positive fibroblastic cells are frequently detected in the fibrotic areas associated with endometriosis. Indeed, immunohistochemical

analyses have shown that endometriotic stromal cells can differentiate to α-SMA-positive myofibroblasts in a process called smooth muscle metaplasia (SMM) (44). Smooth muscle metaplasia is a highly complex cellular process that derives from other processes such as epithelial-mesenchymal transition (EMT) and fibroblast-to-myofibroblast transdifferentiation (FMT) (45). Recent research suggests an important role of activated platelets to promote EMT, FMT and differentiation to SMM in endometriosis (46, 47). Nevertheless, and although much interest has been focused on this matter because of its clinical relevance, the mechanisms by which fibrosis initiates are unclear.

Finally, many investigators have suggested that there is an association between the presence of endometriosis and some kind of immunological dysfunction (48, 49). This theory suggests that changes in cell-mediated and humoral immunity could contribute to the onset and progression of the disease. Although retrograde menstruation is considered a natural event, as it occurs in roughly 90% of women, only 10-15% of women will present endometriosis. In normal conditions, the immune system is able to recognize and scavenge endometrial debris placed in the healthy peritoneum. Nevertheless, in women who develop endometriosis, refluxing endometrial cells may not be destroyed, either due to the abundance of the reflux, to the capacity of some ectopic cells to evade the peritoneal immune system, or to other intrinsic defects of the immune cells. Thus, implantation and proliferation of these ectopic cells occur (50, 51). Hundreds of publications have found alterations in different immune cells of patients with endometriosis. For example, macrophage concentration and proportion are increased in PF of women with the disease. Besides, macrophages are the primary contributors to the elevated proinflammatory and chemotactic

cytokines found in PF of endometriosis patients (52, 53). Regarding cytotoxic T-cell immunity, in human as well as rhesus monkey, the lymphocyte proliferative response to autologous endometrial cells is decreased in endometriosis (54). Besides cytotoxic T lymphocytes, helper T cells also present a diminished activity in PF of women with endometriosis, probably due to higher concentration of putative inhibitory substances (55, 56). Whereas many studies have demonstrated aberrant production of autoantibodies in endometriosis, contradictory reports are found regarding the number and function of B lymphocytes in this disorder (57). What it seems clear is that the activity of these cells may be increased, producing more IgG and IgA (58). These data may be related to another frequent observation in endometriosis, that is the finding of autoantibodies commonly present in other autoimmune diseases, such as antinuclear antibodies, antiDNA antibodies, and antiphospholipid antibodies (59, 60). This suggests that endometriosis may be associated with abnormal policlonal B-cell activation, as it occurs in autoimmune diseases. In fact, many publications have suggested that endometriosis may be considered an autoimmune disease, as it fulfills most of the classification criteria for autoimmune diseases (61, 62). However, there are contradicting results regarding an increased likelihood of other autoimmune diseases in endometriosis patients (63, 64). Apart from all these cell subtypes, probably the most studied group of immune cells in endometriosis patients are natural killer (NK) cells (65). In general, NK cells are directly cytotoxic and they do not obey to laws of specific immunity. They are responsible for rejection of tumors or infected cells by releasing small cytoplasmic granules of proteins that induce apoptosis. Their function is mediated by the balance of activating and inhibiting receptors on cell surface. A possible link between NK cells and endometriosis was initially arisen

from a study which showed the ability of peripheral blood NK cells to destroy endometrial cells (66). Indeed, succeeding studies demonstrated that NK cells cytotoxic activity was reduced in endometriosis, and this reduction correlated with the severity of the disease (67, 68). The next step was to demonstrate the cause for NK cells cytotoxic activity impairment, and some studies suggested the presence of NK inhibiting factors in sera and PF of patients with endometriosis (69, 70). Further experiments demonstrated that supernatants of cultured eutopic endometrial stromal cells from women with endometriosis had a higher inhibitory effect on NK cells cytotoxicity than those without the disease (71, 72). Those findings suggested that substances present in ectopic and eutopic endometrial cells of women with endometriosis have the potential to supress NK cell cytotoxic activity. One of these substances could be the intercellular adhesion molecule 1 (I-CAM). Natural killer cells can recognize aberrant cells when they express I-CAM, however, some neoplastic cells are able to escape form NK surveillance by shedding I-CAM. Soluble I-CAM has been detected in PF of endometriosis patients, and it has been postulated that the molecule was shed from ectopic endometrial cells (73). This could be a mechanism by which ectopic endometrial cells might evade immune surveillance inhibiting NK cells function (74). However, it is possible that besides I-CAM there might be other molecules responsible for the dysfunction of NK cells cytotoxic activity.

1.3. The ADAM17/Notch signalling pathway and its relation with oxidative stress, fibrosis and immune dysfunction

The "A disintegrin and metalloproteinases" (ADAM) family are proteases that cleave membrane-bound proteins and/or degrade the extracellular matrix. ADAMs are closely related to matrix metalloproteinases (MMPs) and twenty-one ADAMs have been described in the human genome, although only 13 of them are proteolytically active (75). ADAMs have tissue-specific functions and promote different physiological processes, that may range from migration and cellular adhesion, to proliferation and fertilization. Indeed, gene targeting of different ADAMs is embryonic lethal, highlighting its importance during individual development. ADAM17, also known as tumor necrosis factor-α converting enzyme (TACE), is the prototype of the ADAM family ectodomain shedding proteases. ADAM-17 is known to cleave a large variety of cell surface proteins, such as cytokines (e.g. TNF-α), cytokine receptors (e.g. IL6-R and TNF-R), ligands (e.g. NKG2DL) and adhesion proteins (e.g. L-selectin and I-CAM) (76). As ADAM17 was first identified as the enzyme that cleaves TNF-α, it was expected that ADAM17 activity would be increased in inflammatory diseases with elevated levels of TNF-α, and it was further demonstrated in rheumatoid arthritis and inflammatory bowel disease (77, 78). More recently, ADAM17 increases have also been associated with the pathogenesis of systemic lupus erithematosus, systemic sclerosis (79, 80), and with the development of many cancers (81, 82). Although, the origins of ADAM17 activity increases are not completely understood, it has recently been shown that oxidative stress is able to induce the synthesis of some members of the ADAMs family, such as ADAM17 (83, 84).

One of the main targets of ADAM17 is Notch receptor (85). In mammals, the Notch family consists of 4 transmembrane receptors (Notch-1 through Notch-4). The binding of Notch to one of its ligands triggers an extracellular cleavage by

ADAM17, thus leading to the formation of a membrane-tethered cleaved form of Notch, which is not the active form of the receptor. This first step is then followed by a second cleavage achieved by a γ-secretase complex that allows the release of the intracellular domain of Notch (NICD), the active form of Notch proteins. NICD translocates to the nucleus where it is involved in the transcriptional regulation of nuclear target genes. The main function of Notch is the regulation of many developmental processes, including proliferation, differentiation and apoptosis (86). Indeed, it has been shown that deregulation of Notch signalling leads to alterations in many tissues, including the vascular and immune systems (87). Besides, changes in Notch signalling can lead to cancer, and current research is focused on this receptor and its therapeutic implications (88, 89). Recent studies have shown that overexpression of Notch signalling may have fibrogenic effects in a wide variety of diseases, including sclerodermia, idiopathic pulmonary fibrosis, kidney fibrosis and cardiac fibrosis (90-92). In a very elegant study, Kavian *et al*. demonstrated a direct link between oxidative stress, the induction of ADAM17, and the activation of Notch pathway and consequent fibrosis in a mouse model of systemic sclerosis (80). Although it is still to be determined, it is possible that this signalling pathway may be initially deregulated by oxidative stress in many other profibrotic disorders.

Interestingly, natural killer group 2, member D (NKG2D) ligands are other targets of ADAM17 protease activity (93). The activating NKG2D receptor is a C-type, lectine-like, type II transmembrane glycoprotein, usually expressed on NK cells and other immune cells. There have been described multiple NKGD2L, which are structural homologs of the *major histocompatibility complex* (MHC) class I molecules. In humans, these ligands are MHC class-I chain related proteins (MIC)

A, MICB and UL16 binding protein (ULBP) 1-6 (94). The binding of NKGD2 receptor to its corresponding ligands triggers cytotoxic effector activity of NK cells and certain T-cell subsets and provides a costimulatory signal for cytokine production. Despite some exceptions, NKG2D ligands (NKG2DL) are not expressed on healthy tissues to avoid inadvertent damage (95). This process is known as the missing self hypothesis. The expression of these ligands may be induced in response to a variety of stimuli related to cellular stress such as heat shock, viral infection, DNA damage, oxidative stress and certain proinflammatory signals (93, 96). Thus, the interaction between NKG2D and its ligands plays an important role in the immunosurveillance of cancer and tumor control. Indeed, some tumor cells have acquired mechanisms to inhibit this system and avoid being recognized by the immune system (97). Cell surface expression of NKG2DL is down-regulated by proteolytic shedding mediated by metalloproteinases that come from oxidative stress (98-100). Consequently, the proteolytic cleavage of NKGD2L from the cell surface by ADAM17 and other proteases is considered an important immune escape mechanism of various cancer cells. Paradoxically, after shedding of NKG2DL, the presence of the soluble form of NKG2DL downmodulates NKG2D expression on NK cells and inhibits T cell activation, thus contributing to tumor immune escape (101). Accordingly, elevated serum levels of soluble NKGD2L have been associated with poor prognosis in several types of cancer (102).

1.4. From basic research to clinics: the relevance of this project

Endometriosis is a chronic benign inflammatory condition that affects 10-15% of women in childbearing age (5). Although some of these women do not present

clinical consequences of the disease, others suffer from pelvic pain, dysmenorrhea and/or infertility. The burden of this disease negatively impacts over the quality of life of millions of women around the world. Despite being described long time ago (22, 103), and after abundant research with thousands of papers published on the topic during the last decades, the question regarding endometriosis pathogenesis is still unresolved. In fact, and although much more is known about molecular aspects of the disease, the main specific treatments for endometriosis are still the same as some decades ago: surgery and/or medical treatments with progestins and other drugs to block endogen estrogens. Medical therapies are not compatible with achieving spontaneous pregnancy as they usually interfere with ovulation. New drugs should be developed that may act locally on endometriotic lesions without interfering with ovarian function and allowing treatment of endometriosis-related pain with an improvement of the odds of natural conception (104, 105).

The studies included in this project are part of a research line on endometriosis pathogenesis. Currently, there is no data that associates oxidative stress, inflammation, stromal cell dysfunction, fibrosis and immune dysfunction in endometriosis. Each one of these aberrations may have its clinical relevance as they may be related to pelvic pain and infertility. Research on the ADAM17/Notch signalling pathway may reveal a common link between these typical characteristics of the disease. Understanding the relationship between the molecular alterations found in endometriosis is crucial to unravel the secrets of its pathogenesis and to find more appropriate and specific treatments for such an enigmatic disorder.

2. HYPOTHESES

Oxidative stress triggers hyperactivation of the ADAM17/Notch signalling pathway, thus stimulating fibrosis production and NKG2DL shedding in endometriosis pathogenesis.

Specific hypotheses

1. Patients with endometriosis have higher levels of oxidative stress and ADAM17 in PF than women without the disease.

2. Endometrial stromal cells of patients with DIE present an increase of ADAM17 levels and NICD compared to endometrial stromal cells of women without the disease.

3. Fibrosis markers are increased in ectopic endometrial stromal cells from endometriosis compared to controls, and γ-secretase inhibitors generate a higher reduction of such fibrosis surrogates in endometrial stromal cells of DIE patients than in controls.

4. Stimulation of endometrial stromal cells with H_2O_2, ADAM17 purified protein and culture with supernatants from endometrial stromal cells of DIE lesions generates a corresponding increase of NICD and fibrosis markers.

5. Soluble PF NKG2DL are increased in women with endometriosis compared to controls, and correlate with the severity of the disease.

3. OBJECTIVES

To demonstrate that patients with endometriosis present an increased oxidative stress status that generates a hyperactivation of the ADAM17/Notch signalling, leading to excessive fibrosis and NKG2DL shedding.

Specific objectives

1. To evaluate oxidative stress and ADAM17 levels in PF of women with and without endometriosis.

2. To assess the levels of ADAM17 and NICD in endometrial stromal cells of patients with DIE and women without the disease.

3. To describe the fibrosis status, evaluated by means of fibrosis markers, and its reduction generated by γ-secretase inhibitors in endometrial stromal cells of DIE patients and controls.

4. To demonstrate the corresponding increase of NICD and fibrosis surrogates after stimulation of endometrial stromal cells with H_2O_2, ADAM17 purified protein and culture with supernatants from endometrial stromal cells of DIE lesions and controls.

5. To evaluate the levels of soluble PF NKG2DL in women with and without endometriosis, and correlate such levels with clinical parameters of the severity of the disease.

4. MATERIAL AND METHODS

To achieve the main and specific objectives, two different studies were planned and performed as explained below.

4.1. Study 1

Dysregulation of the ADAM/Notch signalling pathway in endometriosis: from oxidative stress to fibrosis.

González-Foruria I, Santulli P, Chouzenoux S, Carmona F, Chapron C, Batteux F. Molecular Human Reproduction. 2017 May; 9:1-12.

Study design: Prospective laboratory study.

Study population: Non-pregnant, younger than 42-year-old patients to whom abdominal surgery was performed for a benign gynaecological condition.

Interventions:

- Complete surgical exploration of the abdominopelvic cavity (either by laparotomy or laparoscopy).
- Peritoneal fluid samples acquisition.
- Endometrial biopsy collection from eutopic endometrium and ectopic endometrium of DIE lesions.
- Stromal endometrial cells culture.
- Use of Notch cleavage inhibitors (DAPT and FLI-06).
- Stimulation of stromal endometrial cells with ADAM17 purified protein, increasing concentrations of H_2O_2 and primary cell culture supernatants.

Measures:

a. Surgical diagnosis of endometriosis or other benign gynaecological conditions.

b. Advanced oxidation protein products (AOPP) and metalloproteinase activity of ADAM17 in PF.

c. ADAM17, NICD and fibrosis markers (α-SMA and type-I collagen) in stromal cells from endometrial specimens.

Outcome variables: surgical diagnosis, AOPP, ADAM17, NICD, α-SMA and type-I collagen.

4.2. Study 2

Soluble ligands for the NKG2D receptor are released during endometriosis and correlate with disease severity.

González-Foruria I, Santulli P, Chouzenoux S, Carmona F, Batteux F, Chapron C.

PLOS ONE. 2015; 10(3): e0119961

Study design: Prospective laboratory study.

Study population: Non-pregnant, younger than 42-year-old patients to whom abdominal surgery was performed for a benign gynaecological condition.

Interventions:

- Complete surgical exploration of the abdominopelvic cavity (either by laparotomy or laparoscopy).
- Peritoneal fluid samples collection.

Measures:

a. Surgical diagnosis of endometriosis or other benign gynaecological conditions.

b. Peritoneal fluid soluble NKG2DL (MICA, MICB and ULBP-2)

Outcome variables: surgical diagnosis, MICA, MICB and ULBP-2

4.3. Description of research methodology

4.3.1. Patients

A consecutive series of 202 less than 42-years-old, non-pregnant women who underwent surgery (operative laparoscopy or laparotomy) for a benign gynaecological disorder in a tertiary-care university hospital (Centre Hospitalier Universitaire Cochin, Paris) were recruited for the study between January 2011 and April 2013. Clinical and biological data were prospectively collected in all the patients. Women with cancer and those who did not give their informed consent were excluded from the study. The women were classified into two groups depending on surgical findings (106): the endometriosis group consisted of subjects with histologically-proven endometriosis, while patients in the control group did not show any macroscopic sign of the disease after a meticulous exploration of the abdominal cavity during the surgical procedure.

Endometriosis was staged and scored (total, implant and adhesion scores) according to the revised American Fertility Society (rAFS) Classification (107). In addition, patients with endometriosis were also staged according to the endometriosis phenotype. Based on histological findings, endometriotic lesions were classified into three groups: SUP, OMA and DIE. As these three types of lesions are frequently associated and may coexist (9) patients with endometriosis were classified according to the most severe finding. Endometriotic lesions are usually ranked from the least severe to the most severe in SUP, OMA and DIE (108).

The study analysis used a prospectively managed database. For each patient, personal history data were obtained during face-to-face interviews, which

were conducted by the surgeon the month before surgery. A highly structured previously published questionnaire was used in all patients (109, 110). The following items were recorded: age, parity, gravidity, height, weight, body mass index (BMI) and past history of hormonal and/or surgical treatment for endometriosis, existence of gynaecological pain symptoms (dysmenorrhea, deep dyspareunia, non-cyclic chronic pelvic pain –NCCPP-), gastrointestinal (111) and lower urinary tract symptoms. According to a previous publication, NCCPP is defined as intermittent or permanent pelvic pain not related to the menstrual cycle (112). In order to evaluate the pain intensity preoperatively a previously validated 10-cm visual analog scale was used (113). The use of antigonadotropic oral contraceptives (OC) was recorded in each group and was defined as the use of an OC for at least 6 months before surgery (106, 114).

4.3.2. Collection of PF samples

Peritoneal fluid was taken during surgery from all the patients included in the study (n= 202). The samples were centrifuged at 800g for 10 min at 4ºC and supernatants were collected. Aliquots of the samples were stored at -80ºC until analysis. Peritoneal fluid protein concentration was measured in all the samples using the spectroscopic Bradford protein assay method (115).

4.3.3. Tissue collection, cell isolation and culture

Control endometrial specimens were collected from (n=8) patients without any macroscopic endometriotic lesion as checked during a thorough surgical examination of the abdominopelvic cavity. Indications for surgery in controls were

the following: infertility, fibroids or non-endometriotic ovarian cysts. All samples were histologically characterised for patients and controls (116). Both eutopic and ectopic endometrium were obtained from patients with endometriosis (n=8). In the endometriosis group the ectopic implant consisted in low rectal endometriosis nodules, defined by full-thickness invasion of the muscular layer of the rectum (110).

Primary endometrial and deep endometriotic cell cultures were prepared from biopsies as described by Ngo *et al*. (28). Biopsy specimens were rinsed and minced into small pieces then digested with 5‰ dispase and collagenase (2 mg/ml, Gibco Invitrogen, Cergy Pontoise, France) for 1 h at 37 °C and separated using serial filtration. Red blood cells were removed by hypotonic lysis (0.15 M NH_4Cl, 1 mM $KHCO_3$, 0.1 mM Na_2EDTA). Debris was removed using sieves with 100-μm apertures; epithelial cells were retained on sieves with 40-μm apertures while stromal cells remained in the filtrate. Stromal cells were plated onto Primaria flasks (Becton Dickinson Labware, Le Pont de Claix, France) and cultured in DMEM (Gibco Invitrogen, Cergy Pontoise, France) with 10% FCS. Two populations of cells were obtained from each patient with DIE: eutopic endometrial stromal cells (*Es*), and DIE stromal cells (*Ps*). For each control we used one cell population of eutopic endometrial stromal cells (*Cs*).

The purity of stromal and epithelial cell suspensions was assessed by staining with 1:100 FITC-labeled anti-cytokeratin and 1:100 Cy3-labeled anti-vimentin antibodies (Sigma Aldrich, St Louis, MI, USA). Fluorescence was analysed using an Olympus fluorescent microscope (Hamburg, Germany) and images were captured using the Cell Imaging station (Olympus). Both populations were negative for CD3 (T cells), CD45 (leukocytes) and CD11b (monocytes and

granulocytes) staining. All the experiments were performed on primary cultures of each cell population, and the various tests were performed in triplicate. Primary cultures were obtained between 7 and 14 days after collecting the samples. Oestrogen and progesterone were undetectable in cell culture supernatants as determined by an immunodiagnostic system (Advia Centaur XP, Siemens Health Care Diagnostics, Saint-Denis, France).

4.3.4. Advanced oxidation protein products and ADAM17 metalloproteinase activity in PF

Advanced oxidation protein products were measured as previously described by Witko-Sarsat *et al*. (117). Briefly, 200 µl of PF diluted 1:5 in phosphate-buffered saline were placed into each well of a 96-well microtitre plate. Afterwards, 20 µl of acetic acid were added into each well. For the standards, 10 ml of 1.16 M potassium iodide (Sigma, St Louis, MO, USA) were added to 200 ml of chloramine-T solution (0–100 mmol/l) (Sigma) in each well and then 20 ml of acetic acid was added. The absorbance of the reaction mixture was immediately read at 340 nm against a blank consisting of 200 ml of phosphate-buffered saline, 10 ml of 1.16 M potassium iodine and 20 ml of acetic acid. In order to avoid biases due to PF concentrations or dilutions, a ratio between AOPP (µmol/l) and protein concentrations (mg/ml) was calculated for each peritoneal sample individually (values are expressed in nmol/mg).

The Mca-PLAQAV-Dpa-RSSSR-NH2 Fluorogenic Peptide Substrate III (R&D Systems, Inc., Minneapolis, MN, USA, Catalog # ES003) was used for the assay of ADAM17 activity. Fluorogenic peptide substrate III was used to measure the

activities of peptidases that are capable of cleaving an amide bond between the fluorescent group and the quencher group, causing an increase in fluorescence. The peptide sequence is derived from the pro-tumour necrosis factor-α (pro-TNF-α). This peptide acts as substrate for TNF-α converting enzyme (TACE/ADAM17). Fluorogenic peptide substrate III (50 µl) was directly added to 50 µl of PF for 1 hour at 37°C. Fluorescence was recorded after 60 minutes on a spectrofluorimeter (Fusion; Packard, USA) at 320 nm and 405 nm as excitation and emission wavelengths, respectively. The fluorescence intensity strongly reflects the ADAM17 enzyme activity (118). A ratio between the ADAM17 fluorescence result (pg/ml) and the protein concentration (mg/ml) was calculated for each peritoneal sample individually (values are expressed in pg/mg).

4.3.5. Immunoblotting measurement of ADAM17, activated Notch and α-SMA in endometrial cells

Equal amounts of proteins (40 µg) were loaded and separated by 10% SDS-PAGE. Transfer and blocking were performed. Polyacrylamide membranes were saturated with 5% skimmed milk for 1 h at room temperature and incubated overnight at 4°C with Cleaved Notch-1 (Val 1744) Antibody (Cell Signaling Techonology, Danvers, MA, USA) and Human ADAM17 Antibody (R&D Systems, Inc., Minneapolis, MN, USA), respectively. The membranes were then washed and specific antibodies were detected using a 1:1000 dilution of horseradish peroxidase-conjugated goat anti-rabbit IgG Abs (Dako, Denmark) and visualized by an Enhanced Chemiluminescence system (Advansta, California, USA). After film exposure, the membranes were washed three times for 10 min each in TBS/T.

Membranes were incubated for 30 min at 50 °C in stripping buffer (100 mM β-mercaptoethanol, 2% sodium dodecyl sulfate, 62.5 mM Tris–HCl, pH 6.7). Afterwards, they were washed three more times during 10 min each in TBS/T. Membranes were saturated with 5% skimmed milk for 1 h at room temperature and then incubated with 1:50 000 of a peroxydase labelled monoclonal anti β-Actin antibody (Sigma) 1 hour at room temperature and visualized by an Enhanced Chemiluminescence system (Advansta, Menlo Park, California, USA). Optical densities were measured using Multigauge Software (Fujifilm). A ratio between ADAM17 optical density or Notch optical density and β-Actin optical density was calculated in each case (arbitrary units are reflected).

Similarly, the expression of α-SMA was assessed using Western blot with an anti-mouse α-SMA antibody (clone 1A4; Sigma). Specific antibodies were detected using a horseradish peroxidase-conjugated goat anti-rabbit IgG antibody (Dako, Denmark) and visualized by an enhanced Chemiluminescence system (Advansta, Menlo Park, California, USA). Expression was calculated as a ratio between α-SMA optical density divided by β-Actin (Sigma) optical density (arbitrary units are reflected).

4.3.6. Effect of Notch cleavage inhibition by DAPT and FLI-06 on α-SMA expression and type-I collagen production in endometrial cells

Endometrial stromal cells from ectopic nodules of DIE patients (*Ps* cells) and endometrial stromal cells from control patients (*Cs* cells) were incubated with either 100 μ M DAPT (N-S-phenyl-glycine-t-butyl ester) (Sigma, St Louis, USA) or 2.3μM FLI-06 (Selleckchem, Houston, USA), both being γ-secretase inhibitors.

4.3.7. Cleaved Notch and α-SMA expression in endometrial cells after stimulation with ADAM17 purified protein, increasing concentrations of H_2O_2 and culture supernatants

Additionally, *Cs* and *Ps* cells were also incubated with H_2O_2 at increasing concentrations ranging from 0µM to 40µM or stimulated with *Cs* and *Ps* cells culture supernatants or incubated with ADAM17 purified protein (0.01µg/ml) (R&D system northeast Minneapolis, USA). For each condition, cells were cultured during 24 hours at 37°C.

4.3.8. Collagen content assay

After 24 hours of treatment with DATP (100µM) and FLI-06 (2.3µM), the samples were stained with 1% Picrosirius red for 1 hour and cells were then washed three times with acetic acid (0.5% in H_2O). Staining was eluted using sodium hydroxide and quantified at 540 nm optical density. Type-I collagen was evaluated as arbitrary units per million cells.

4.3.9. Measurement of soluble NKG2DL concentration

MICA, MICB and ULBP-2 were assayed in the PF by an enzyme-linked immunosorbent assay (ELISA) (R&D Systems, Inc., Minneapolis, MN, USA), according to the manufacturer's recommendations. The range of determination for MICA and ULBP-2 was 62'5-4.000 pg/ml and for MICB 156-10.000 pg/ml. The results below the lower threshold levels were considered as 0 pg/ml for the statistical analysis. Each sample was tested in duplicate and reflected the mean of

the two measurements. In order to avoid biases owing to PF concentration or dilution at the moment of obtaining the sample, a ratio between the NKG2DL result (pg/ml) and the protein concentration (mg/ml) was calculated for each peritoneal sample individually (values are expressed in pg of NKG2DL / mg of protein).

4.3.10. Statistical analysis

Data were analysed using GraphPad Prism 5 Software (GraphPad Software, Inc., San Diego, CA, USA) and Statistical Package for the Social Sciences Software (SPSS Inc., Chicago, IL, USA). The Student's t-test was used for quantitative variables and the Pearson's Chi-square or Fisher's exact test for qualitative variables as appropriate. Considering the non-Gaussian distribution of our biological parameters, statistical analysis was performed between two groups using non-parametrical statistical tests. All quantitative data are expressed as the median (range). Analyses between two groups were performed using the Mann-Whitney non-parametric U test. When the analysis included more than 2 groups, the Kruskal-Wallis test was used. The post-hoc tests were performed using Dunn's Multiple Comparison test. The non-parametric Spearman correlation test was used to assess correlations. P values less than 0.05 were considered significant.

Comparison of α-SMA and collagen in both groups of cells was performed using a non-parametric test for matched samples (Wilcoxon matched pairs test).

In the view of the number of samples with undetectable levels of MICA, MICB and ULBP-2, two different statistical analyses were performed, one including and the other excluding the samples with undetectable levels of these NKG2DL.

5. RESULTS

5.1. Patients and controls

One hundred twenty-one women with endometriosis and 81 disease-free women were included in the study. The patients' clinical and surgical characteristics are shown in Table 1.

Following the endometriosis surgical classification (7) and based on the location of the worst lesion presented, the 121 histologically proven endometriotic patients were classified as follows: 41 (33.9%) SUP, 32 (26.4%) OMA (right 11; left 14; bilateral 7) and 48 (39.7%) DIE. Patients' distribution according to the worst DIE lesion found was the following: 13 (27.1%) uterosacral ligament(s), 7 (14.6%) vagina, 5 (10.4%) bladder, 21 (43.7%) intestine and 2 (4.2%) ureter. These 48 DIE patients presented a total of 113 histologically proven DIE lesions distributed as follows: 46 uterosacral ligament lesions, 15 vaginal lesions, 7 bladder lesions, 43 intestinal lesions (1 intestinal lesion in 10 patients and more than 1 intestinal lesion in 13 patients) and 2 ureteral lesions. The mean (± SD) number of DIE lesions per patient was 2.3 ± 1.5 (range 1-6).

Surgery indications in the 81 endometriosis-free women were the following: uterine fibroids (29 patients, 35.8%), non-endometriotic benign ovarian cysts (21 patients, 25.9%), tubal infertility (15 patients, 18.6%), pelvic pain (7 patients, 8.6%) and other indications (9 patients, 11.1%).

No differences were detected in age, gravidity, parity and infertility between the endometriosis group and control group. The BMI was significantly lower in endometriotic patients than controls (P= 0.001). The percentage of patients with preoperative hormonal treatment was similar between the two groups (Table 1).

Patient characteristics	Endometriosis (*N* = 121)	Controls (*N* = 81)	*P*
Age (years)[a]	30.8 ± 5.1	31.7 ± 5.36	0.267[t]
Height (cm)[a]	167.5 ± 6.1	164.1 ± 6.0	<0.001[t]
Weight (kg)[a]	59.5 ± 8.1	61.5 ± 9.9	0.162[t]
BMI (kg/m^2)[a]	21.2 ± 2.5	22.8 ± 3.4	0.001[t]
Parity[a]	0.2 ± 0.5	0.4 ± 0.7	0.093[t]
Gravidity[a]	0.4 ± 0.7	0.6 ± 1.0	0.123[t]
Preoperative hormonal treatment (*n*, %)	48 (40.0%)	33 (40.1%)	0.887[k]
Previous treatment for endometriosis			
Hormonal treatment (*n*, %)*	68 (56.2%)	NA	
Previous surgery (*n*, %)	19 (15.7%)	NA	
Previous endometrioma's surgery (*n*, %)	11 (9.1%)	NA	
rAFS classification			
Mean implants score rAFS[a,b]	11.3 ± 11.2	NA	
Mean adhesions score rAFS[a,b]	9.6 ± 16.3	NA	
Mean total score rAFS[a,b]	21.2 ± 23.1	NA	
rAFS stage (*n*, %)[b]		NA	
I	37(30.6%)		
II	16 (13.2%)		
III	26 (21.5%)		
IV	42 (34.7%)		
Surgical classification			
SUP (*n*, %)	41 (33.9%)	NA	
Endometrioma (*n*,%)	32 (26.4%)	NA	
Endometrioma laterality (*n*, %)		NA	
Bilateral	7/32 (21.9%)		
Right	11/32 (34.4%)		
Left	14/32 (43.7%)		
DIE lesions (*n*, %)[c]	48 (39.7%)	NA	

Table 1. Baseline characteristics of participants

5.2. AOPP and ADAM17 levels in PF

AOPP and ADAM17 were measured in PF from all the study population (n=202). The levels of AOPP were significantly increased in endometriotic patients compared to controls (median, 1.73 nmol/mg; range, 0.43-411.9 vs. median, 1.25 nmol/mg; range, 0.27-18.26; P< 0.001) (Fig. 1A). According to the surgical classification, AOPP levels were different among groups (P< 0.01). A post-hoc test showed a significant increase in PF AOPP levels in DIE patients (which represent the most severe forms of the disease) as compared to controls (median, 2.22 pg/mg; range, 0.63-411.9 vs. median, 1.30 pg/mg; range, 0.28-18.26; P< 0.01) (Fig. 1B).

Women with endometriosis presented a significantly higher activity of ADAM17 metalloproteinase than women without the disease (median, 0.28 pg/mg; range, 0.06-469.6 vs. median, 0.20 pg/mg; range, 0.07-52.7; P= 0.019) (Fig. 1C). According to the surgical classification, ADAM17 levels were significantly different among groups (P< 0.05). The post-hoc test revealed increased ADAM17 levels in DIE patients as compared to controls (median, 0.34 pg/mg; range, 0.06-469.6 vs. median, 0.20 pg/mg; range, 0.07-52.7; P< 0.05) (Fig. 1D).

In addition, a significant biological correlation was found between the AOPP peritoneal levels of patients with endometriosis and ADAM17 activity (r= 0.614; P< 0.001) (Fig. 1E).

In order to rule out any bias concerning the effect of preoperative hormonal treatment, we compared AOPP and ADAM17 levels in patients who used hormonal treatment with patients who did not. We failed to show any differences in any of the biomarkers according to the use of OC (P= 0.934 and P= 0.406 for AOPP and ADAM17, respectively).

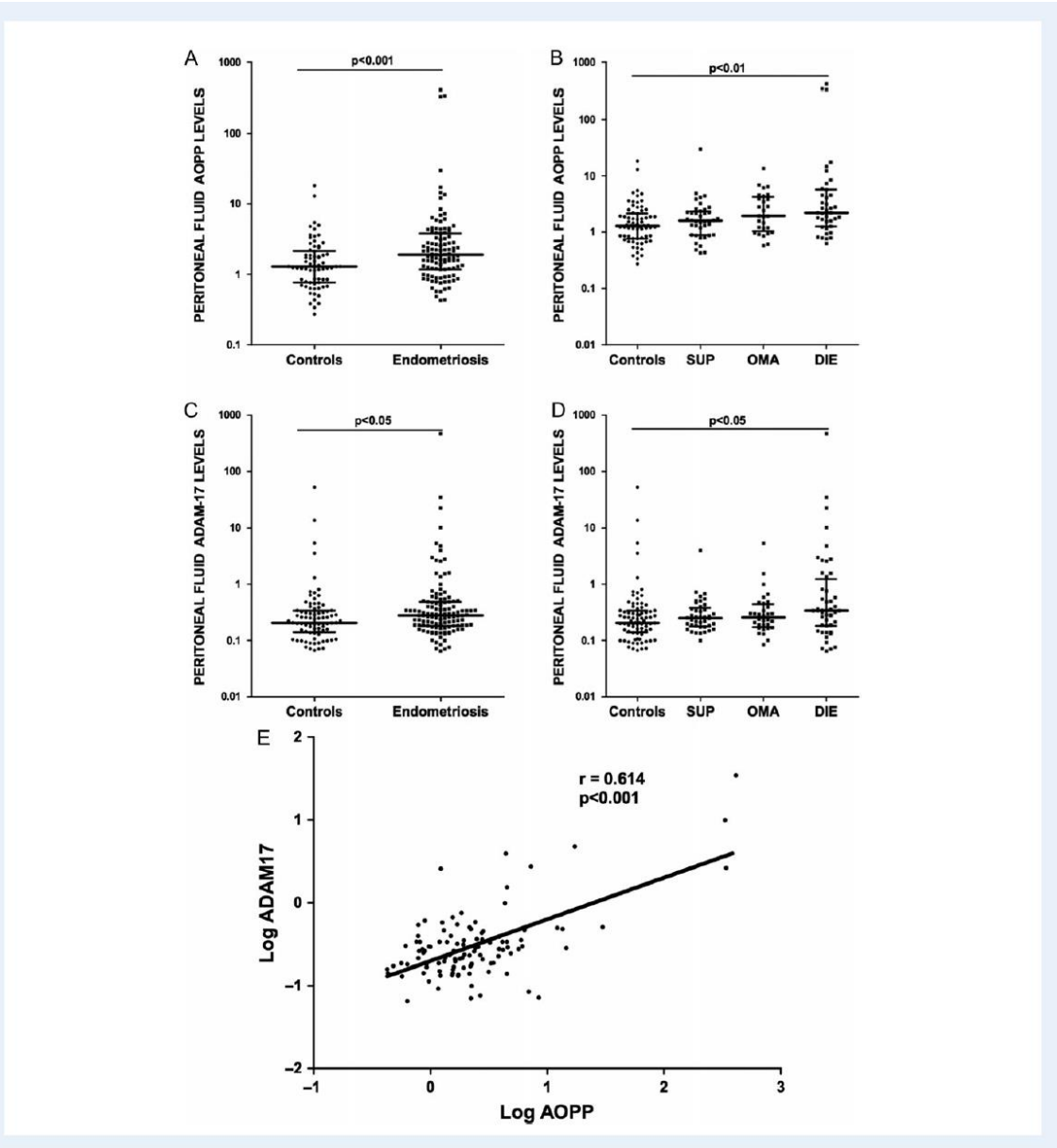

Figure 1. AOPP and ADAM17 levels in patients with endometriosis and controls

5.3. ADAM17 and Notch activity in stromal endometrial cells

ADAM17 protein expression was assessed by Western blot in all the different primary cell cultures derived from *Cs* of control patients and *Es* and *Ps* endometrium of DIE patients (Fig. 2A).

ADAM17 expression in *Cs*, *Es* and *Ps* cells was significantly different among groups (*Cs* mean optical density, 0.11; SEM ± 0.02 vs. *Es* mean, 0.63; SEM ± 0.10 vs.

Ps mean, 0.53; SEM ± 0.07; P< 0.001). The post-hoc test showed increased ADAM17 activity in *Es* and *Ps* cells with respect to *Cs* cells (P< 0.001 and P< 0.01 respectively) (Fig. 2A).

Notch activation was evaluated using Western blot by measuring the amounts of Notch intracellular domains (NICD) contained in the different primary cell cultures derived from *Cs*, *Es* and *Ps* cells. Higher amounts of NICD were shown in stromal endometrial cells from DIE patients in comparison with disease-free patients (*Cs* mean optical density, 0.17; SEM ± 0.03 vs. *Es* mean, 0.34; SEM ± 0.10 vs. *Ps* mean, 0.39; SEM ± 0.08; P= 0.040). A post-hoc test revealed a significant increase of Notch signalling in *Ps* compared to *Cs* cells (P< 0.05). The differences observed between *Cs* cells and *Es* cells did not reach statistical significance (Fig. 2B).

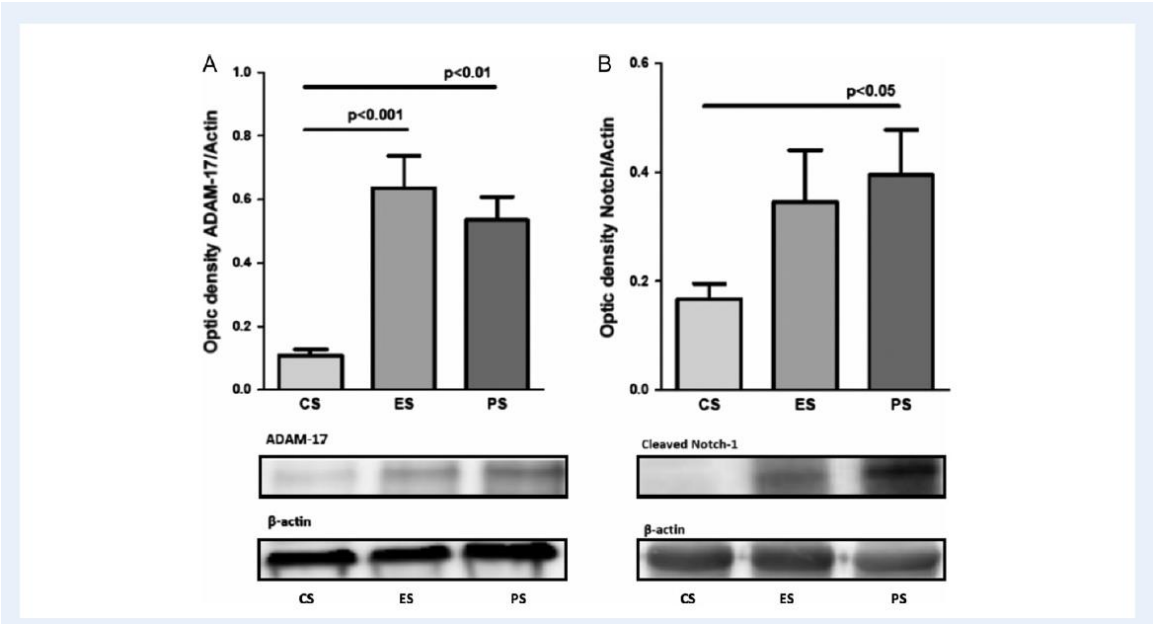

Figure 2. ADAM17 and Notch-1 intracellular domain (NICD) levels on stromal endometrial cells of study patients

5.4. Fibrosis markers evaluation in ectopic stromal endometrial cells from DIE (*Ps*) and eutopic stromal endometrial cells from controls (*Cs*) before and after Notch cleavage inhibition

Basal levels of α-SMA and type-I collagen were significantly increased in *Ps* compared to *Cs* cells (*Ps* mean optical density for α-SMA, 0.80; SEM ± 0.14 vs. *Cs* mean, 0.54; SEM ± 0.10; P= 0.009; *Ps* mean optical density for type-I collagen, 0.53; SEM ± 0.07 vs. *Cs* mean, 0.34; SEM ± 0.03; P= 0.015) (Fig. 3).

After treatment with DAPT and FLI-06 (both γ-secretase inhibitors that prevent Notch cleavage and NICD release) *Ps* cells presented a significant reduction of α-SMA (*Ps* untreated mean optical density, 0.80; SEM ± 0.14 vs. *Ps* DAPT mean, 0.45; SEM ± 0.14; P= 0.006; *Ps* untreated vs. *Ps* FLI-06 mean, 0.47; SEM ± 0.17; P= 0.031) (Fig. 3A and 3B) and type-I collagen expression (*Ps* untreated mean optical density, 0.53; SEM ± 0.07 vs. *Ps* DAPT mean, 0.39; SEM ± 0.02; P= 0.048; *Ps* untreated vs. *Ps* FLI-06 mean, 0.37; SEM ± 0.06; P= 0.039) (Fig. 3C). Notwithstanding, no differences were observed in α-SMA levels when *Cs* cells with normal amounts of NICD were treated with DAPT and FLI-06, (*Cs* untreated mean optical density, 0.54; SEM ± 0.10 vs. *Cs* DAPT mean, 0.48; SEM ± 0.07; P= 0.276; *Cs* untreated vs. *Cs* FLI-06 mean, 0.45; SEM ± 0.11; P= 0.149)(Fig. 3A and 3B) or type-I collagen (*Cs* untreated mean optical density, 0.34; SEM ± 0.03 vs. *Cs* DAPT mean, 0.33; SEM ± 0.04; P= 0.999; *Cs* untreated vs. *Cs* FLI-06 mean, 0.32; SEM ± 0.04; P= 0.808) (Fig. 3C).

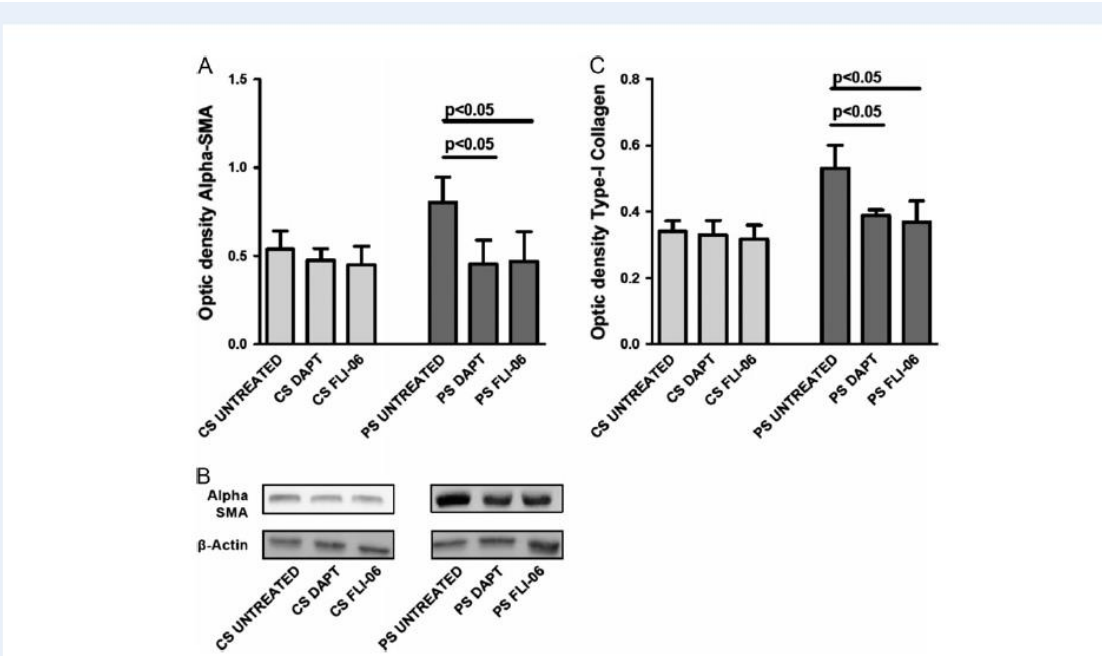

Figure 3. Evaluation of fibrosis markers before and after treatment with γ-secretase inhibitors

5.5. Cleaved Notch and α-SMA expression after stimulation with ADAM17 purified protein, increasing concentrations of H_2O_2 and culture supernatants

Stimulation with ADAM17 purified protein generated a significant increase in cleaved Notch both in *Cs* (*Cs* untreated mean optical density, 0.20; SEM ± 0.06 vs. *Cs* + ADAM17 mean, 0.56; SEM ± 0.04; P= 0.013) and *Ps* cells (*Ps* untreated mean optical density, 0.60; SEM ± 0.17 vs. *Ps* + ADAM17 mean, 1.38; SEM ± 0.21; P= 0.001) (Fig. 4A), It is noteworthy that the increase was much higher in *Ps* cells. Similarly, ADAM17 stimulation augmented α-SMA expression in *Cs* (*Cs* untreated mean optical density, 0.42; SEM ± 0.02 vs. *Cs* + ADAM17 mean, 0.75; SEM ± 0.08; P= 0.016) and *Ps* cells (*Ps* untreated mean optical density, 0.81; SEM ± 0.09 vs. *Ps* + ADAM17 mean, 1.60; SEM ± 0.13; P= 0.002) (Fig. 4B).

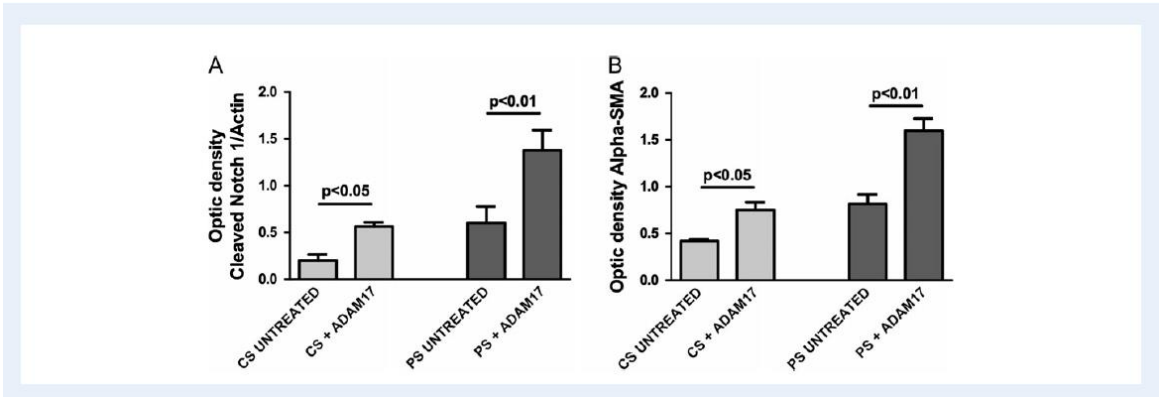

Figure 4. Evaluation of Notch-1 intracellular domain and α-SMA levels before and after stimulation with ADAM17 purified protein

Furthermore, when stromal endometrial cells were incubated with increasing concentrations of H_2O_2, both *Cs* and *Ps* cells showed a progressive

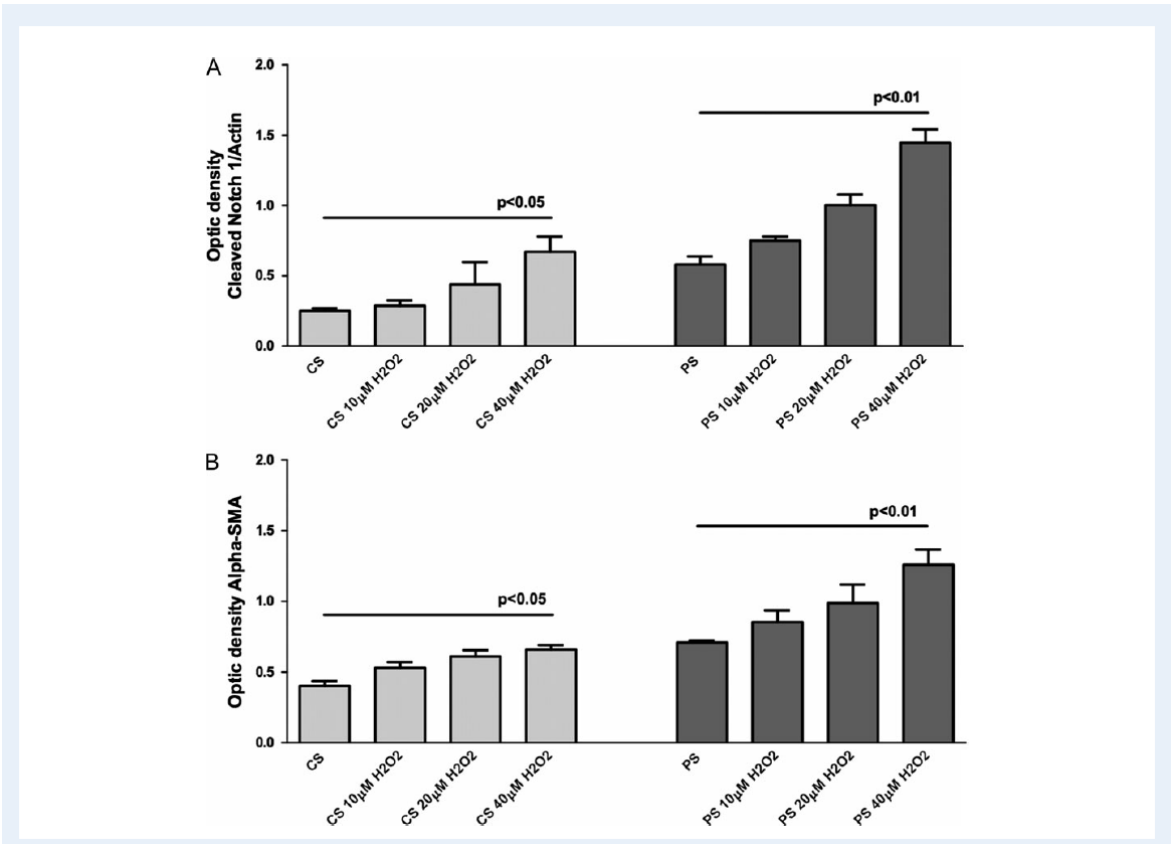

Figure 5. Evaluation of Notch-1 intracellular domain and α-SMA levels before and after incubation with increasing concentrations of hydrogen peroxide

increase in cleaved Notch levels (P< 0.05 and P <0.01 for *Cs* and *Ps* cells, respectively) (Fig. 5A) and in α-SMA expression (P< 0.05 and P< 0.01) (Fig. 5B).

In order to evaluate if *Cs* and *Ps* cell culture supernatants were sufficient to induce an increase of Notch and α-SMA, both types of cells were incubated with their counterpart supernatant and vice-versa. Rising Notch concentrations were observed when *Cs* and *Ps* cells were treated with *Ps* supernatants. The post-hoc test showed a significant increase of Notch expression in *Cs* cells treated with *Ps* supernatant (*Cs* untreated mean optical density, 0.26; SEM ± 0.08 vs. *Cs* + *Ps* supernatant mean, 0.81; SEM ± 0.24; P= 0.036) (Fig. 6A), and in *Ps* cells treated with *Ps* supernatant compared to their basal untreated cells (*Ps* untreated mean optical density, 0.69; SEM ± 0.07 vs. *Ps* + *Ps* supernatant mean, 1.75; SEM ± 0.05; P= 0.003) (Fig. 6B). No differences in Notch were observed when *Cs* and *Ps* cells were treated with *Cs* supernatant. Accordingly, α-SMA assessment revealed differences in its levels when both *Cs* and *Ps* cells were incubated with *Ps* supernatants. The post-hoc test analysis revealed a significant increase in α-SMA expression in *Cs* cells treated with *Ps* supernatant (*Cs* untreated mean optical density, 0.44; SEM ± 0.06 vs. *Cs* + *Ps* supernatant mean, 0.84; SEM ± 0.11; P= 0.028) (Fig. 6C), and in *Ps* cells incubated with their own *Ps* supernatant (*Ps* untreated mean optical density, 0.69; SEM ± 0.12 vs. *Ps* + *Ps* supernatant mean, 2.75; SEM ± 0.36; P= 0.005)(Fig. 6D). Notwithstanding, when *Cs* and *Ps* cells were treated with *Cs* supernatant, no significant increase in α-SMA expression was observed.

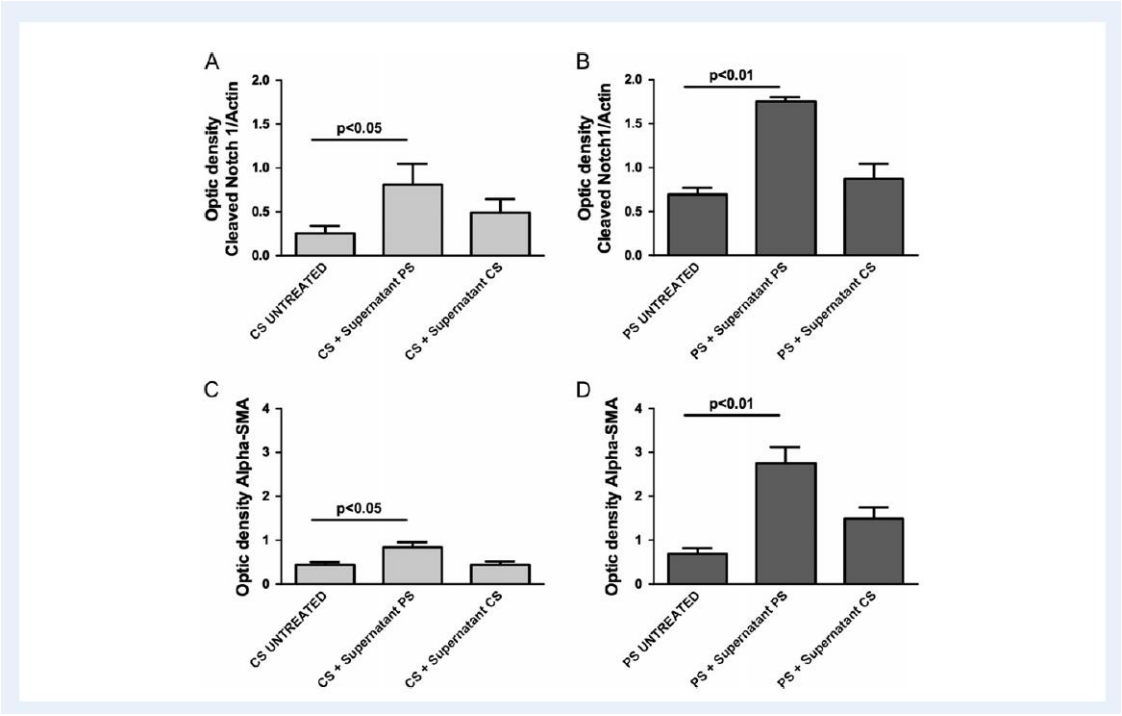

Figure 6. Evaluation of Notch-1 intracellular domain and α-SMA levels before and after incubation with supernatants

5.6. MICA, MICB and ULBP-2 levels in PF of study participants

MICA, MICB and ULBP-2 levels were measured in PF of all the patients included in the study (n= 202). MICA, MICB and ULBP-2 were detected in 140 (69.3%), 196 (97.0%) and 34 (16.8%) study participants respectively.

MICA was detected in 82 (67.8%) endometriosis-affected women and in 58 (71.6%) controls (P= 0.641). MICB resulted positive in 118 (97.5%) patients with endometriosis and in 78 (96.3%) endometriosis-free patients (P= 0.685). Detection rate for ULPB-2 was significantly higher in endometriotic women as compared to controls (27 (22.3%) vs. 7 (8.6%), respectively; P= 0.012) (Table S1). According to the surgical classification, among endometriosis-affected women, MICA was detected in 25 (60.9%) SUP, 24 (75.0%) OMA and 33 (68.7%) DIE patients (P= 0.566). MICB became positive in 39 (95.1%) SUP, 31 (96.8%) OMA

and 48 (100%) DIE patients (P= 0.543), while ULBP-2 was detected in 7 (17.1%) SUP, 10 (31.2%) OMA, 10 (20.8%) DIE patients (P= 0.026) (Table S1).

When samples with undetectable PF levels of MICA, MICB and ULBP-2 were excluded, MICA and MICB levels were significantly higher in endometriosis patients than in controls (median, 1.1 pg/mg; range, 0.1-143.5 vs. median, 0.6 pg/mg; range, 0.1-3.5; P= 0.003 for MICA; median, 4.6 pg/mg; range, 1.2-4702 vs. median, 3.4 pg/mg; range, 0.7-20.1; P= 0.001 for MICB). In contrast, ULPB-2 levels in endometriosis patients were not significantly different from controls (median, 0.4 pg/mg; range, 0.1-5.2 vs. median, 0.5 pg/mg; range, 0.1-4.2; P= 0.551). In a similar way, when considering all the samples of endometriosis patients and controls, the levels of MICA (median, 0.5 pg/mg; range, 0.0-143.5 vs. median, 0.2 pg/mg; range, 0.0-3.5; P= 0.060), MICB (median, 4.9 pg/mg; range, 0.0-4702 vs. median, 3.4 pg/mg; range, 0.0-20.1; P< 0.001) and ULBP-2 (median, 0.0 pg/mg; range, 0.0-5.2 vs median, 0.0 pg/mg; range, 0.0-4.2; P= 0.014) resulted higher in the endometriosis group than in the control group, although for MICA the differences did not reach statistical significance (Table 2 and Figure 7).

Table 2. Statistical analyses for peritoneal NKG2D ligands (MICA, MICB and ULBP-2) ratio levels in women with endometriosis and controls.

xTable 2: NKG2D Ligands ratio levels in women with endometriosis and controls.

	Endometriosis	Controls	p
Peritoneal MICA (with)	(n = 121) 0.48 (0.0–143.5)	(n = 81) 0.23 (0.0–3.5)	0.060 [u]
Peritoneal MICA (without)	(n = 82) 1.06 (0.1–143.5)	(n = 58) 0.56 (0.1–3.5)	0.003 [u]
Peritoneal MICB (with)	(n = 121) 4.87 (0.0–4702)	(n = 81) 3.40 (0.0–20.1)	<0.001 [u]
Peritoneal MICB (without)	(n = 118) 4.61 (1.2–4702)	(n = 78) 3.48 (0.7–20.1)	0.001 [u]
Peritoneal ULBP-2 (with)	(n = 121) 0.00 (0.0–5.2)	(n = 81) 0.00 (0.0–4.2)	0.014 [u]
Peritoneal ULBP-2 (without)	(n = 27) 0.36 (0.1–5.2)	(n = 7) 0.52 (0.1–4.2)	0.551 [u]

Table 2. Peritoneal NKGD2L (MICA, MICB and ULBP-2) levels in women with endometriosis and controls

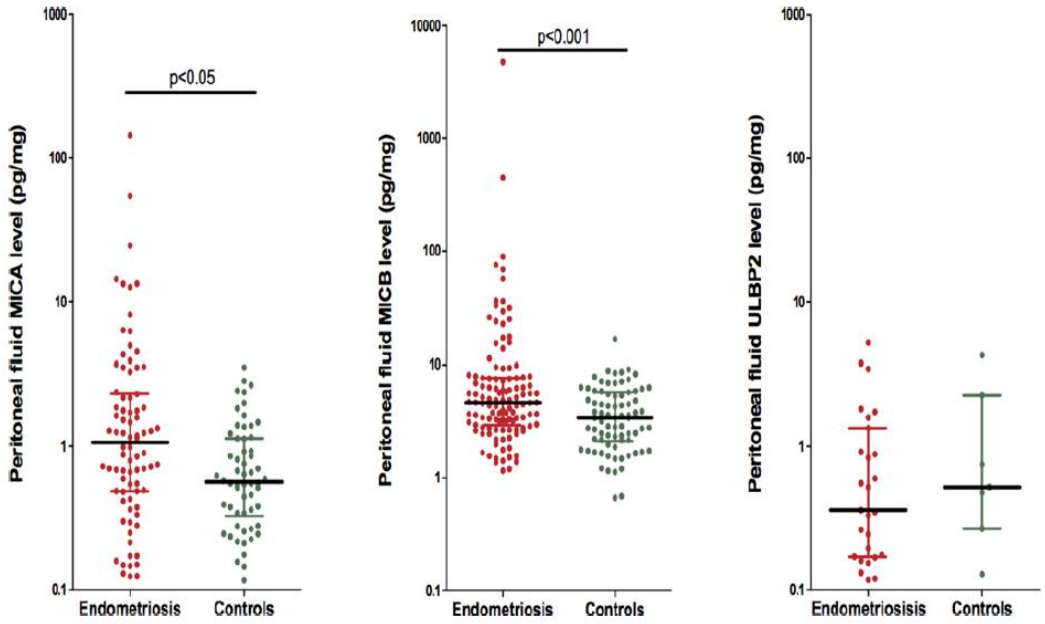

Figure 7. Peritoneal NKG2DL in endometriosis and controls

According to the surgical classification, the medians of detectable MICA, MICB and ULBP-2 levels in DIE, OMA, SUP and control patients are depicted in Figure 8. MICA (P= 0,015), MICB (P= 0.002) and ULBP-2 (P= 0.045) levels were significantly different between groups. A post-hoc test showed a significant increase in peritoneal MICA and MICB levels in DIE patients with the most severe forms of the disease compared to controls (median, 1.2 pg/mg; range, 0.1-143.5 vs. median, 0.6; range, 0.1-3.5; P< 0.05; median, 5.0 pg/mg; range, 1.4-4702 vs median, 3.6 pg/mg; range, 0.7-20.1; P< 0.01 for MICA and MICB respectively). In addition MICB levels were also significantly increased in SUP patients versus controls (median, 5.6 pg/mg; range, 1.2-35.1 vs. median, 3.6 pg/mg; range, 0.7-20.1; P <0.05). When post-hoc test was performed for ULBP-2 there were significant differences between DIE and SUP patients (median, 0.9 pg/mg; range, 0.2-5.2 vs. median, 0.2 pg/mg; range, 0.1-0.5; P< 0.05).

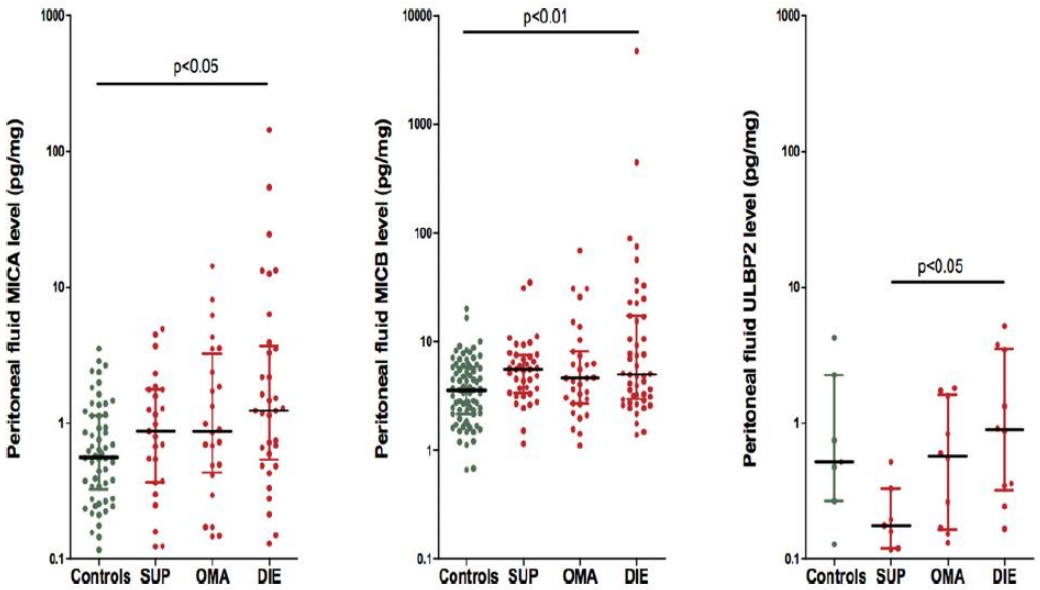

Figure 8. Peritoneal fluid NKG2DL levels in patients with endometriosis and controls according to the surgical classification of endometriosis

When all the samples were included in the analysis according to the surgical classification, MICA levels did not show differences between the groups (P= 0.100). However, MICB (P= 0.001) and ULBP-2 (P= 0.026) peritoneal levels were significantly different among groups. The post-hoc test showed for MICB significantly increased values in DIE and SUP patients compared to controls (median, 5.0 pg/mg; range, 1.4-4702 vs. median, 3.4 pg/mg; range, 0.0-20.1; P< 0.01; median, 5.4 pg/mg; range, 0.0-35.1 vs. median, 3.4 pg/mg; range, 0.0-20.1; P< 0.05 for DIE and SUP patients respectively). The same analysis for ULBP-2, showed higher values in the OMA group in comparison to controls (median, 0.0 pg/mg; range, 0.0-1.8 vs. median, 0.0 pg/mg; range, 0.0-4.2; P <0.05) (Table S2).

5.7. Clinical correlations with PF MICA, MICB and ULBP-2

Clinical, surgical and biological correlations with PF MICA, MICB and ULBP-2 levels in women with endometriosis are expressed in Table S3.

MICA correlated with MICB (r= 0.466; P <0.001) and ULBP-2 (r= 0.540; P= 0.009). In addition, MICA presented a clinical correlation with dysmenorrhea (r= 0.232; P= 0.029). There was also a positive correlation between MICA and Total rAFS score (r= 0.221; P= 0.031) and with Adhesions rAFS score (r= 0.221; P= 0.031). ULBP-2 levels also correlated with Adhesions rAFS score (r= 0.217; P= 0.034).

MICA correlated not only biologically with other NKG2DL, but also clinically with pain score and surgically with some surgical findings corresponding to the severity of the disease. MICA correlations with MICB, ULBP-2, Total rAFS score and Dysmenorrhea are depicted in Figure S2.

6. DISCUSSION

This work provides evidence that oxidative stress, the ADAM17/Notch signalling pathway and NKG2DL shedding phenomenon are associated and play a role in the pathogenesis of endometriosis. The results of these studies show that increased oxidative stress present in PF of endometriosis patients is correlated with augmented levels of ADAM17. Increased ADAM17 activity generates higher NICD concentration after its proteolytic release from the Notch-1 receptor, and consequently, higher fibrosis. Further analyses performed in this study help to reinforce the association between the ADAM17/Notch signalling pathway and fibrosis production in endometriosis. Besides, the finding of increased soluble NKG2DL in PF of patients with endometriosis, especially in those with DIE, may partially explain the NK cell dysfunction present in this disease. Such discoveries suggest a molecular association between chronic inflammation, stromal cell dysfunction, fibrosis and immune impairment and open opportunities to find new potential therapeutic targets in endometriosis.

It is nowadays accepted that endometriosis is an inflammatory disease (5). According to previous publications (28, 119), oxidative stress is altered in endometriotic patients, and plays a crucial role in the onset and progression of the disorder. In **Study 1**, the levels of AOPP, which reflect the intensity of oxidative stress (120), were measured in PF and found significantly elevated in women with endometriosis, especially in patients with DIE, confirming previous observations (27). A significant positive correlation was found between AOPP peritoneal levels of patients with endometriosis and ADAM17 proteinase levels. These levels were also significantly increased in patients with endometriosis compared to controls. According to the surgical classification, AOPP levels, and the highest ADAM17 levels were found in the PF of patients with DIE, the most severe form of

endometriosis. At cellular levels, ADAM17 activity was significantly increased in stromal cells of ectopic (*Ps*) and eutopic (*Es*) endometrium of DIE patients compared to eutopic stromal endometrial cells (*Cs*) of control patients.

Previous *in vitro* experiments have provided evidence that ADAM17 also plays a prominent role in the Notch signalling pathway, during the proteolytic release of the NICD (from the Notch-1 receptor) that occurs following ligand binding (85). In **Study 1**, the highest amounts of NICD were found in *Ps* and *Es* from DIE patients compared to *Cs* cells from disease-free patients. Recent research has shown that Notch signalling is crucial in endometrial decidualization and receptivity, and that both the loss and gain of Notch function may result in implantation impairment (121). The same work attributed Notch with an interesting role in the regulation of progesterone receptor methylation, leading to a progesterone resistance profile. These findings that reveal Notch overexpression in *Es* and *Ps* cells from DIE patients compared to controls may present a relationship with progesterone resistance and the worse reproductive outcomes observed in patients with endometriosis. According to these results, it may be suggested that drugs selectively inhibiting ADAM17 or Notch cleavage (122) could be used to improve reproductive results in these patients. However, this was not the main target of our research and further studies are needed to draw valid conclusions regarding this specific point.

Fibrosis is also a key feature of endometriosis, as this process is significantly associated with the most severe forms of endometriosis, especially DIE and observed in ectopic lesions (39). Indeed, in **Study 1**, the fibrosis markers α-SMA, and type-I collagen were significantly increased in *Ps* cells from DIE patients compared with *Cs* cells from controls. To evaluate the magnitude of the

role of Notch signalling in the fibrotic process that occurs in *Ps* cells from DIE patients, *Ps* and *Cs* cells were treated with DAPT and FLI-06, inhibitors of the γ-secretase complex that prevent Notch cleavage (122, 123). After treatment with these compounds, *Ps* cells presented a significant reduction of α-SMA and type-I collagen, while *Cs* cells with normal amounts of NICD did not show a decrease in either of the two fibrosis markers. These results reveal a hyperactivation of the Notch pathway in ectopic cells from DIE lesions, but not in endometrial cells from controls. For this reason, we believe that the Notch signalling pathway plays an important role in the fibrotic processes of DIE ectopic lesions as already observed in other pro-fibrotic disorders (124). This observation not only provides better understanding of the molecular mechanisms that take place in DIE lesions, but also opens new perspectives for therapeutic interventions such as with γ-secretase inhibitors that are currently under study in clinical trials (125).

In order to reinforce the relationship between ADAM17, Notch and fibrosis in endometriosis, *Ps* and *Cs* cells were stimulated with increasing concentrations of H_2O_2, ADAM17 purified protein and culture with their counterpart supernatant and vice-versa. These additional experiments in **Study 1** showed that each intervention was able to increase Notch and α-SMA regardless of the origin of the cells, although this increase was more evident in *Ps* cells from DIE patients than controls (*Cs*).

The main strength of **Study 1** is based on the novelty of the topic and its accurate methodological design: (I) as far as we are aware, this is the first study in endometriosis patients that relates oxidative stress with the ADAM17/Notch signalling pathway and consequent fibrosis. (II) Moreover, and according to the

heterogeneity of the disease, patients were selected with well-defined clinical phentoypes. Only patients with complete surgical evaluation of the peritoneal cavity were included. Thus, the endometriosis state was perfectly assessed, and patients were allocated to the endometriotic or the control group according to a previously validated classification (7). (III) On the other hand, control patients were evaluated in the same way as endometriotic patients, and therefore, clinical and histopathological findings were also recorded and taken into account. In addition, the diversity of the pathologies present in our control patients reflects the most common benign conditions in gynaecology.

Nonetheless, **Study 1** may have some limitations and biases that should be taken into account: (I) the elevated proportion of patients with severe endometriosis in the study group is not a real representation of the prevalence of this condition among the general population. This selection bias occurs because the recruitment was performed in a highly specialized centre dealing with severe endometriosis. Nevertheless, we believe this consideration does not alter the main outcomes of the study. (II) Although the control population presented the most common benign gynaecological conditions, it is not known whether these disorders may modify oxidative stress and the ADAM17/Notch pathways. (III) Despite the fact that 40% of the patients were under preoperative hormonal treatment, data on the menstrual phases of the rest of the study participants was not available. However, it has been previously demonstrated that AOPP levels do not differ between menstrual phases (27). Additionally, we analysed the effect of hormonal treatment in AOPP and ADAM17 expression, and we failed to find any differences in AOPP and ADAM17 levels between patients who used hormonal treatment and those who did not.

The same population (n= 202) was used in **Study 2**, in order to evaluate soluble NKG2DL in PF. Previous research has shown that the immune system actively contributes to the homeostasis of the peritoneal cavity (48, 49, 126), and that NK cell play a pivotal role in the scavenging of the refluxed endometrial debris in the peritoneum. NKG2DL are not expressed in most normal healthy cells, but they are frequently overexpressed in infected or transformed cells, acting as a threating signal that enables the immune system to recognize stressed cells (95). The liberation of soluble forms of the ectodomain of NKG2DL not only decreases the expression of the ligands on target cells but also generates the internalization and lysosomal degradation of NKG2D receptor, leading to a paradoxical inhibition of NK cells function. In fact, NKG2DL shedding has been shown to be a tumor escape mechanism. In a similar way to tumors, endometriosis avoids immune surveillance in different manners. The findings obtained in **Study 2** demonstrate that MICB levels were significantly elevated in PF of patients with endometriosis as compared to controls, especially in those patients with the most severe forms of the disease. Concerning MICA levels, the endometriosis group presented higher levels of this ligand than controls, though when considering all the samples (detectable and undetectable values), the differences did not reach statistical significance (P= 0.06). The same results could not be obtained for ULBP-2. A strong biological correlation was found between MICA and other NKG2DL (MICB and ULBP-2). In addition, significant correlations were also found between MICA and clinical characteristics of the disease. High PF levels of MICA were positively correlated with dysmenorrhea, total rAFS score and adhesions rAFS score (107), though these clinical associations had a moderate correlation coefficient.

Study 2 demonstrates an increase in soluble forms of NKG2DL in PF of endometriosis patients, suggesting a lower expression of these ligands in ectopic endometrial cells surface, heading toward greater evasion from NK cells recognition. Additionally, the rise in soluble NKG2DL levels would further inhibit NK cells cytotoxicity, and consequently NK cell dysfunction becomes more pronounced. According to this rationale, peritoneal invasion and proliferation of ectopic endometrial cells turns out to be more facile when NKG2DL shedding occurs.

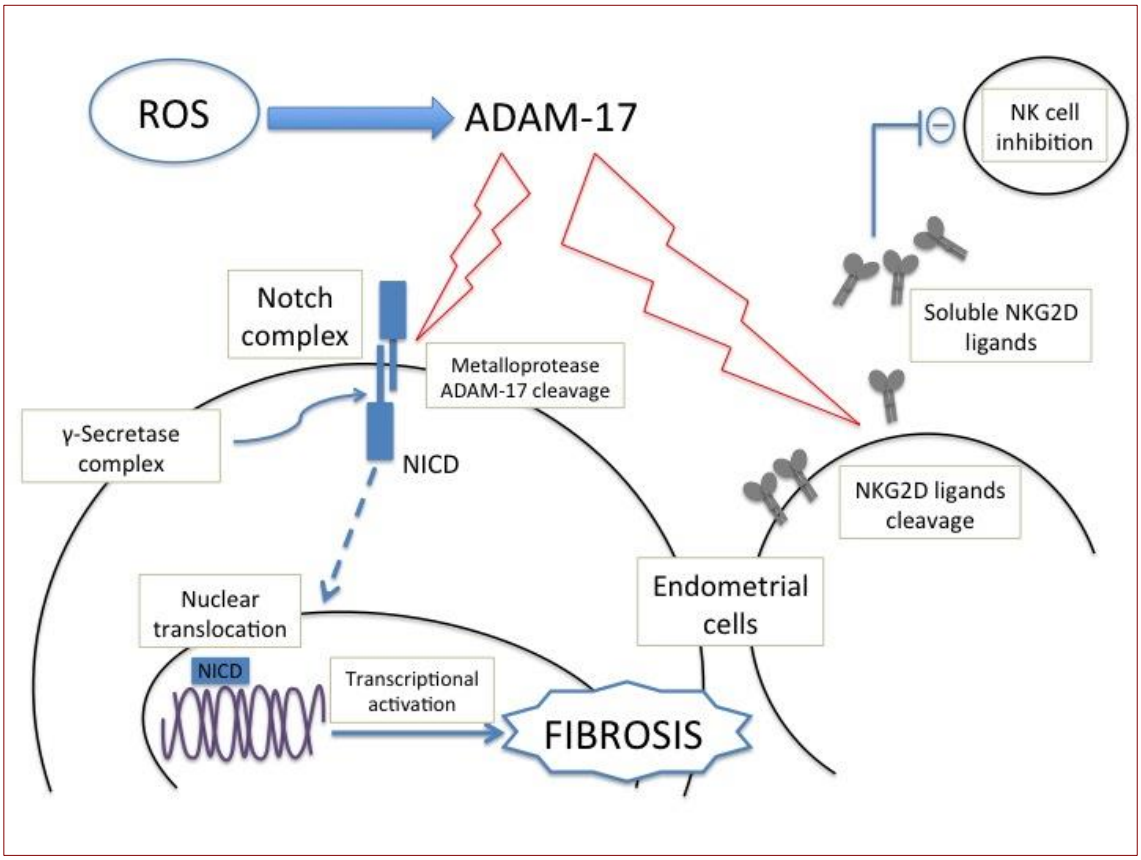

Figure 9. Representation of the relationship between oxidative stress, ADAM17 activity, Notch cleavage, fibrosis and the NKG2DL shedding phenomenon in endometriosis pathogenesis

The clinical correlations observed in this study reinforce even more the aforementioned hypothesis. In this regard, the origin of soluble NKG2DL increase could be explained by the elevated oxidative stress and ADAM17 levels present in endometriosis patients as demonstrated in **Study 1**. As it has already been shown in some cancers (100), the higher oxidative stress exhibited in endometriosis would trigger an elevation of some metalloproteinases, such as ADAM17. A part from the consequences of ADAM17 on Notch-1 receptor and fibrosis, it would also increase the shedding of NKG2DL from ectopic endometrial cells membranes, leading to NK cells dysfunction.

Study 2 presents some major strengths that deserve to be commented: (I) to the best of our knowledge, this is the first study on the assessment of PF NKG2DL levels in endometriotic patients. A large number of PF (n= 202) from patients who were submitted to a gynaecological intervention (121 endometriotic women and 81 controls) were evaluated. (II) Taking into consideration the heterogeneity of the disease (108), patients were selected with very well-defined clinical phenotypes. The inclusion of patients in the study was performed following a thorough abdominopelvic cavity exploration. All surgical and histological findings were recorded in order to grade the severity of the disease and to distribute each patient according to the former described classification (7). (III) Control patients, who exhibited the most common benign conditions in gynaecology, were clinically and surgically evaluated in the same manner as endometriotic patients.

Despite an accurate study design, the results of **Study 2** may be subject to certain shortcomings: (I) although detection rates for MICA and MICB in PF using the ELISA Kit were high (69 and 97% respectively), the rate of detection for ULBP-

2 resulted far below from the others (17%). This high rates of undetectable levels, hampers the statistical analysis of data and its interpretation. The rate of undetectable ULBP-2 levels is a result in itself, though the way these undetectable results should be managed for statistical analysis is not clear so far. For this reason, and in order to avoid possible weak points related to the presence of undetectable levels of ULBP-2, MICA and MICB, we carried out two separated statistical assessments: one including and the other excluding the undetectable values as it has already been done in other studies (37). Nevertheless, despite having a low detection rate for ULBP-2, when a detectable value of this ligand was obtained, the likelihood of belonging to an endometriotic patient was significantly higher. These findings lead us to believe an existing role of ULBP-2 in the pathogenesis of the disease. (II) The high proportion of women with severe endometriosis in our studies, does not reflect the real prevalence of disease severity among general population. This selection bias occurs because patients were recruited in a referral centre specialized in the care of grave endometriosis. However, this consideration should not alter the main results of the study. (III) As it has already been acknowledged in **Study 1**, despite the control population used in these studies display the most common benign gynaecological disorders, it is not clear whether these conditions may modify PF NKG2DL levels. For this reason, the results of **Study 2** must be interpreted with regard. (IV) The MICA ELISA assay may not equally detect all the different soluble MICA molecules; in fact, more than 60 allelic variants have been described. The finding that soluble MICA could be detected in endometriotic and control patients suggests that this system was applicable for a cohort of endometriotic patients. As specific allelic variants of MICA exist, it cannot be ruled out that the differences observed between groups do

not reflect various concentrations of different soluble MICA variants. Thus, special caution should be paid for the use of this ELISA system for widely polymorphic MICA. (V) The ULBP-2 ELISA used in this study exhibits some cross-reactivity with another member of the UL16 binding protein family (ULBP), ULBP-6. Unfortunately, there are no strictly ULBP-2-specific or ULBP-6-specific ELISA available due to the high sequence identity between both ligands, so it cannot be excluded that soluble ULBP-6 has been detected along with soluble ULBP-2 in these study participants.

The results of both studies (**Study 1** and **Study 2**) allow to reach the conclusions that will be revised straightaway in the following section and that correspond with the 5 objectives proposed in the respective section of this PhD thesis (page 39).

7. CONCLUSIONS

1. Oxidative stress, measured by means of Advanced Oxidation Protein Products (AOPP) in peritoneal fluid (PF) is increased in endometriosis patients, especially in those with deep infiltrating endometriosis (DIE). Likewise, ADAM17 activity is elevated in those patients, and presents a significant positive correlation with AOPP levels.

2. Both ADAM17 and Notch intracellular domain (NICD) levels are augmented in eutopic and ectopic endometrial stromal cells from DIE patients, compared to the levels found in eutopic endometrial stromal cells from controls.

3. Ectopic endometrial stromal cells from patients with DIE present higher levels of fibrosis (measured by α-SMA and type-I collagen) than eutopic endometrial stromal cells from women without endometriosis. Moreover, the use of γ-secretase inhibitors generated a significant reduction of fibrosis only in ectopic endometrial cells from DIE patients, revealing a hyperactivation of the Notch pathway in endometriosis.

4. Stimulation of both endometrial stromal cells from DIE and controls with H_2O_2, ADAM17 purified protein and supernatants from the culture of endometrial stromal cells of DIE lesions generates a consequent increase of NICD and fibrosis surrogates. Nevertheless, stimulation with supernatant from endometrial stromal cells from controls does not modify NICD or fibrosis.

5. NKG2DL are elevated in PF of patients with endometriosis, mainly in those with DIE, and show moderate correlations with some clinical parameters.

As a consequence of what has been described, and as a final remark, it can be concluded that increased oxidative stress alters the ADAM17/Notch

signalling pathway and leads to augmentation of fibrosis markers production and NKG2DL shedding in endometriosis pathogenesis.

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9. ACKNOWLEDGEMENTS

My most sincere gratitude to both of my thesis directors. To Paco, my mentor during the last years, who did everything that was in his hands to support my research stay in Paris, and who transmitted me his passion for endometriosis. To Prof. Chapron, who accepted me in his Department in Paris and without whom, all this project would not had been possible.

I would also like to thank all the co-authors in this project. I will always be in debt with Pietro and Frédéric, my spiritual guides in Paris, who despite having thousands of things to do, they always found a moment for helping me in the development of these works. To Sandrine, who showed me how to survive in the lab, and who was always with me during my stay.

I cannot forget the support and help that I have received during all these years from my colleagues at Hospital Clínic and also at Dexeus. Daily work is much easier if you spend time with people you love and who make you smile.

I would obviously like to dedicate a few words to my beloved family. To my parents, who taught me which are the most important things in life, and who have been devoted to me so many years. And finally, to my wife Rebeca, who accepted to come to Paris in a delicate moment of our lives, and who has always supported me during the length of this thesis work.

10. ANNEXES

Figures and Tables Legends:

Table 1. Baseline characteristics of participants

[a] Data are presented as mean ± SD;

[b] Score according to the American Fertility Society Classification (107)

[c] According to a previously published surgical classification for deeply infiltrating endometriosis (DIE) (7)

[t] Student's t-test;

[k] Pearson's chi-square test;

* <5% missing data

NA: not applicable

Figure 1. AOPP and ADAM17 levels in patients with endometriosis and controls: (**A**) Peritoneal fluid Advanced Oxidation Protein Products (AOPP) measured by ELISA in patients with endometriosis (n=121) and in controls (n=81) after exploration of the abdominopelvic cavity. (**B**) AOPP levels measured by ELISA according to the surgical classification of endometriosis [controls (n=81); SUP (n=41); OMA (n=32) and DIE (n=48)]. (**C**) ADAM17 levels measured by ELISA in PF of endometriosis patients and controls. (**D**) ADAM17 levels according to the surgical classification of endometriosis. (**E**) Correlation analysis of peritoneal fluid AOPP and ADAM17 proteinase levels in 121 patients with endometriosis. The logarithmic transformation of peritoneal fluid AOPP and ADAM17 levels is depicted.

Statistical analyses were performed using the Mann-Whitney non-parametric U test for figures A and C, and the Kruskal-Wallis test for figures **B** and **D**. The results of pairwise comparisons using Dunn's Multiple Comparison test are presented (**B**, **D**). The non-parametric Spearman's correlation test was used to assess

correlations (Figure **E**). ADAM17 and AOPP values are represented on a logarithmic scale as a scatter dot plot. ADAM17 values are expressed in pg/mg. AOPP values are expressed in nmol/mg. The medians with their interquartile range are reported.

Figure 2. ADAM17 and Notch-1 intracellular domain (NICD) levels on stromal endometrial cells of study patients: Differences in ADAM17 activity (**A**) and Notch-1 intracellular domain (NICD) levels (**B**) among eutopic stromal endometrial cells (*Cs*) (n=8) of control patients, stromal cells of eutopic (*Es*) (n=8) and ectopic (*Ps*) endometrium of DIE patients (n=8) assessed by Western blot of cell lysates. Statistical analyses were performed using the Kruskall-Wallis test. Dunn's Multiple Comparison test was used for post-hoc analysis. The mean optical density ratio for ADAM17/β-Actin and Notch/β-Actin was calculated for each endometriotic cell type and compared with control endometrial cells. Error bars represent SEM. Arbitrary units are expressed.

Figure 3. Evaluation of fibrosis markers before and after treatment with γ-secretase inhibitors: Evaluation of fibrosis markers (α-SMA and type-I collagen) in eutopic stromal endometrial cells of control patients (*Cs*) and ectopic stromal cells of DIE patients (*Ps*) before and after treatment with γ-secretase inhibitors (DAPT and FLI-06) assessed by Western Blot of cell lysates for α-SMA (**B**) and by picro-sirius red staining for type-I collagen. The mean optical density ratio for α-SMA/β-Actin was calculated in each group of cells (**A**). The mean optical density at 540 nm was used for type-I collagen testing and calculated in each group of cells (**C**). Statistical analyses were performed using a non-parametric test for matched

samples (Wilcoxon matched pairs test). Error bars represent SEM. Arbitrary units are expressed.

Figure 4. Evaluation of Notch-1 intracellular domain and α-SMA levels before and after stimulation with ADAM17 purified protein: Evaluation of Notch-1 intracellular domain (**A**) and α-SMA levels (**B**) in stromal endometrial cells (*Cs*) (n=8) of control patients and ectopic (*Ps*) endometrium of DIE patients (n=8) before and after stimulation with ADAM17 purified protein. Comparisons were performed using statistics for matched samples as appropriate. The mean optical density ratio for Notch/β-Actin and α-SMA/β-Actin was calculated for both cell types. Error bars represent SEM. Arbitrary units are expressed.

Figure 5. Evaluation of Notch-1 intracellular domain and α-SMA levels before and after incubation with increasing concentrations of hydrogen peroxide: Evaluation of Notch-1 intracellular domain (**A**) and α-SMA levels (**B**) in stromal endometrial cells (*Cs*) (n=8) of control patients and ectopic (*Ps*) endometrium of DIE patients (n=8) in basal status and after incubation with increasing concentrations of H_2O_2. Statistical analyses were performed using Friedman test. Dunn's Multiple Comparison test was used for post-hoc analysis. The mean optical density ratio for Notch/β-Actin and α-SMA/β-Actin was calculated for both cell types. Error bars represent SEM. Arbitrary units are expressed.

Figure 6. Evaluation of Notch-1 intracellular domain and α-SMA levels before and after incubation with supernatants: Comparison of Notch-1 intracellular domain (**A** and **B**) and α-SMA levels (**C** and **D**) in stromal endometrial cells (*Cs*)

(n=8) of control patients and ectopic (*Ps*) endometrium of DIE patients (n=8) before and after incubation with their own supernatant and their counterpart supernatant. Statistical analyses were performed using the Friedman test. Dunn's Multiple Comparison test was used for post-hoc tests. The mean optical density ratio for Notch/β-Actin and α-SMA/β-Actin was calculated for both cell types. Error bars represent SEM. Arbitrary units are expressed.

Table 2. Peritoneal NKGD2L (MICA, MICB and ULBP-2) levels in women with endometriosis and controls: Analyses were performed both including samples with undetectable levels of NLG2D ligands (i.e. below lower limit of detection of the assay) and without these samples.

Results are expressed as median (range) . Values are expressed in pg/mg

[u] Statistical analysis was performed with the Mann-Whitney U Test.

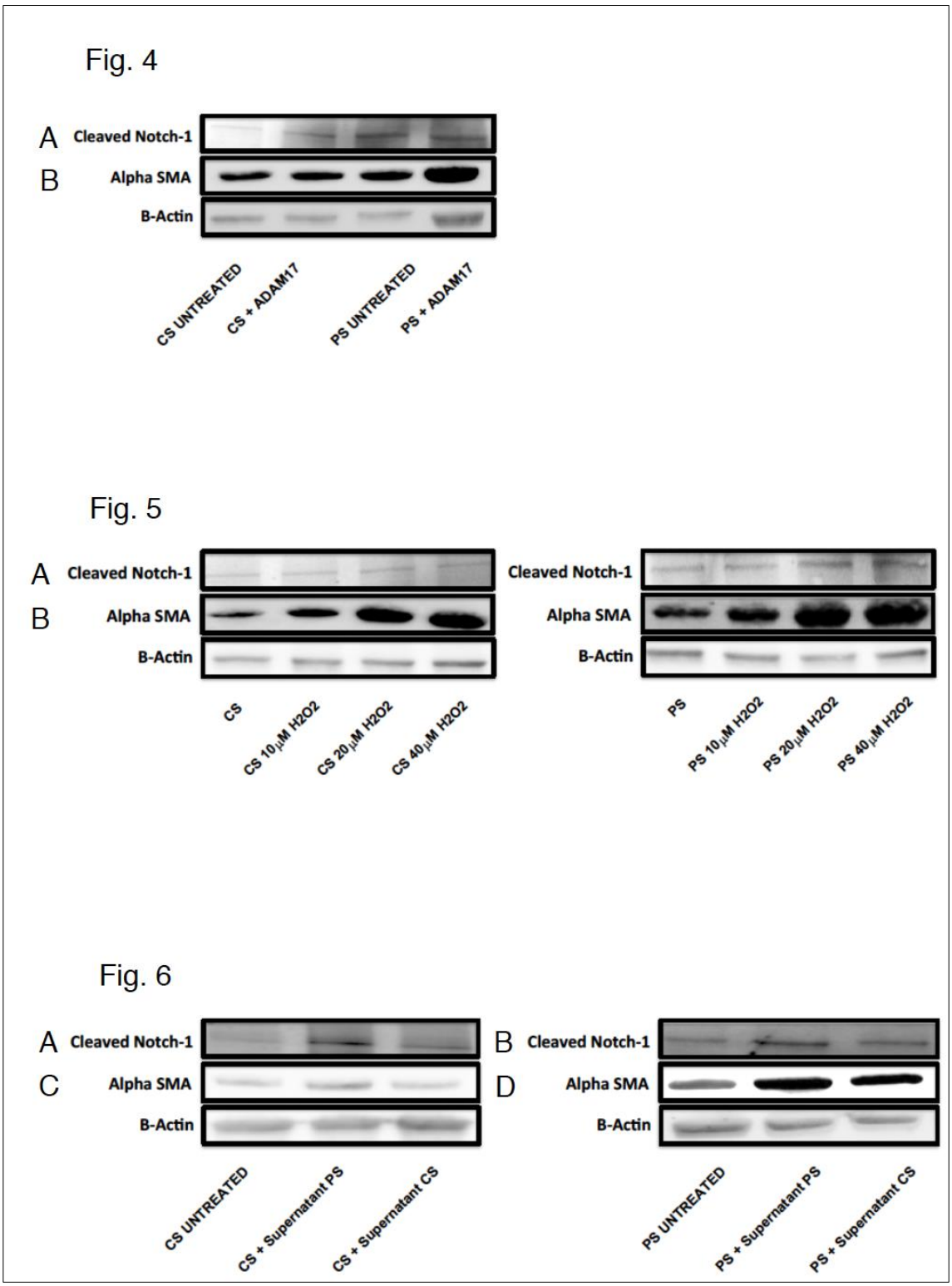
Figure 7. Peritoneal NKG2DL in endometriosis and controls. Samples with undetectable levels of NKG2D ligands have been excluded. The peritoneal fluid MICA and MICB ratio levels among groups were significantly different by the Mann Whitney test (P=0.035 and P<0.001 respectively). Peritoneal NKG2D ligands ratio values are represented on a logarithmic scale as a scatter dot plot. The medians with their interquartile range are reported.

Figure 8. Peritoneal fluid NKG2DL levels in patients with endometriosis and controls according to the surgical classification of endometriosis: Samples with undetectable levels of NKG2D ligands have been excluded. The peritoneal fluid MICA and MICB ratio levels among groups [DIE (n=48), OMA (n=31); SUP

(n=39) and controls (n=78)] were significantly different by the Kruskal-Wallis test (P=0.015 and P=0.002 respectively). Post hoc test were performed using the with Dunn's Multiple Comparison Test. Peritoneal NKG2D ligands ratio values are represented on a logarithmic scale as a scatter dot plot. The medians with their interquartile ranges are reported.

Figure 9. Representation of the relationship between oxidative stress, ADAM17 activity, Notch cleavage, fibrosis and the NKG2DL shedding phenomenon in endometriosis pathogenesis

<u>Figure S1</u>. Representative Western Blot of Figures 4, 5 and 6.



<u>Table S1</u>. NKG2D Ligands qualitative results according to the surgical classification.

MICA	POSITIVE (n, %)	NEGATIVE (n, %)	p
SUP (n=41)	25 (61)	16 (39)	0.566 [k]
OMA (n=32)	24 (75)	8 (25)	
DIE (n=48)	33 (69)	15 (31)	
Controls (n=81)	58 (72)	23 (28)	

MICB	POSITIVE (n, %)	NEGATIVE (n, %)	p
SUP (n=41)	39 (95)	2 (5)	0.543 [k]
OMA (n=32)	31 (97)	1 (3)	
DIE (n=48)	48 (1)	0 (0)	
Controls (n=81)	78 (96)	3 (4)	

ULBP-2	POSITIVE (n, %)	NEGATIVE (n, %)	p
SUP (n=41)	7 (17)	34 (83)	0.026 [k]
OMA (n=32)	10 (31)	22 (69)	
DIE (n=48)	10 (21)	38 (79)	
Controls (n=81)	7 (9)	74 (91)	

MICA	POSITIVE (n, %)	NEGATIVE (n, %)	p
ENDO (n=121)	82 (68)	39 (32)	0.64 [f]
Controls (n=81)	58 (72)	23 (28)	

MICB	POSITIVE (n, %)	NEGATIVE (n, %)	p
ENDO (n=121)	118 (97)	3 (3)	0.68 [f]
Controls (n=81)	78 (96)	3 (4)	

ULBP-2	POSITIVE (n, %)	NEGATIVE (n, %)	p
ENDO (n=121)	27 (22)	94 (78)	0.01 [f]
Controls (n=81)	7 (9)	74 (91)	

[k] Pearson's chi-square test;

[f] Fisher exact test;

<u>Table S2</u>. Peritoneal NKG2DL levels according to the surgical classification. Analyses were performed both including samples with undetectable levels of NKG2D ligands (i.e. below lower limit of detection of the assay) and without these samples.

	DIE	OMA	SUP	Controls	p
Peritoneal MICA (with)	(n=48) 0.6 (0.0-143.5)	(n=32) 0.6 (0.0-14.3)	(n=41) 0.2 (0.0-4.9)	(n=81) 0.2 (0.0-3.5)	0.100 [k]
Peritoneal MICA (without)	(n=33) 1.2 (0.1-143.5)*	(n=24) 0.9 (0.1-14.3)	(n=25) 0.9 (0.1-4.9)	(n=58) 0.6 (0.1-3.5)	0.015 [k]
Peritoneal MICB (with)	(n=48) 5.0 (1.4-4702)**	(n=32) 4.5 (0.0-68.7)	(n=41) 5.4 (0.0-35.1)*	(n=81) 3.4 (0.0-20.1)	0.001 [k]
Peritoneal MICB (without)	(n=48) 5.0 (1.4-4702)**	(n=31) 4.6 (1.1-68.7)	(n=39) 5.6 (1.2-35.1)*	(n=78) 3.6 (0.7-20.1)	0.002 [k]
Peritoneal ULBP-2 (with)	(n=48) 0.0 (0.0-5.2)	(n=32) 0.0 (0.0-1.8)*	(n=41) 0.0 (0.0-0.5)	(n=81) 0.0 (0.0-4.2)	0.026 [k]
Peritoneal ULBP-2 (without)	(n=10) 0.9 (0.2-5.2)***	(n=10) 0.6 (0.1-1.8)	(n=7) 0.2 (0.1-0.5)	(n=7) 0.5 (0.1-4.2)	0.045 [k]

Results are expressed as median (range)

Values are expressed in pg/mg

SUP: superficial peritoneal endometriosis

OMA: Endometrioma

DIE: Deep infiltrating endometriosis

k. Statistical analysis was performed using Kruskal-Wallis test. Post hoc test were performed using the with Dunn's Multiple Comparison Test..

*Significantly different from control women ($p < 0.05$)

** Significantly different from control women ($p < 0.01$)

***Significantly different from SUP ($p < 0.05$)

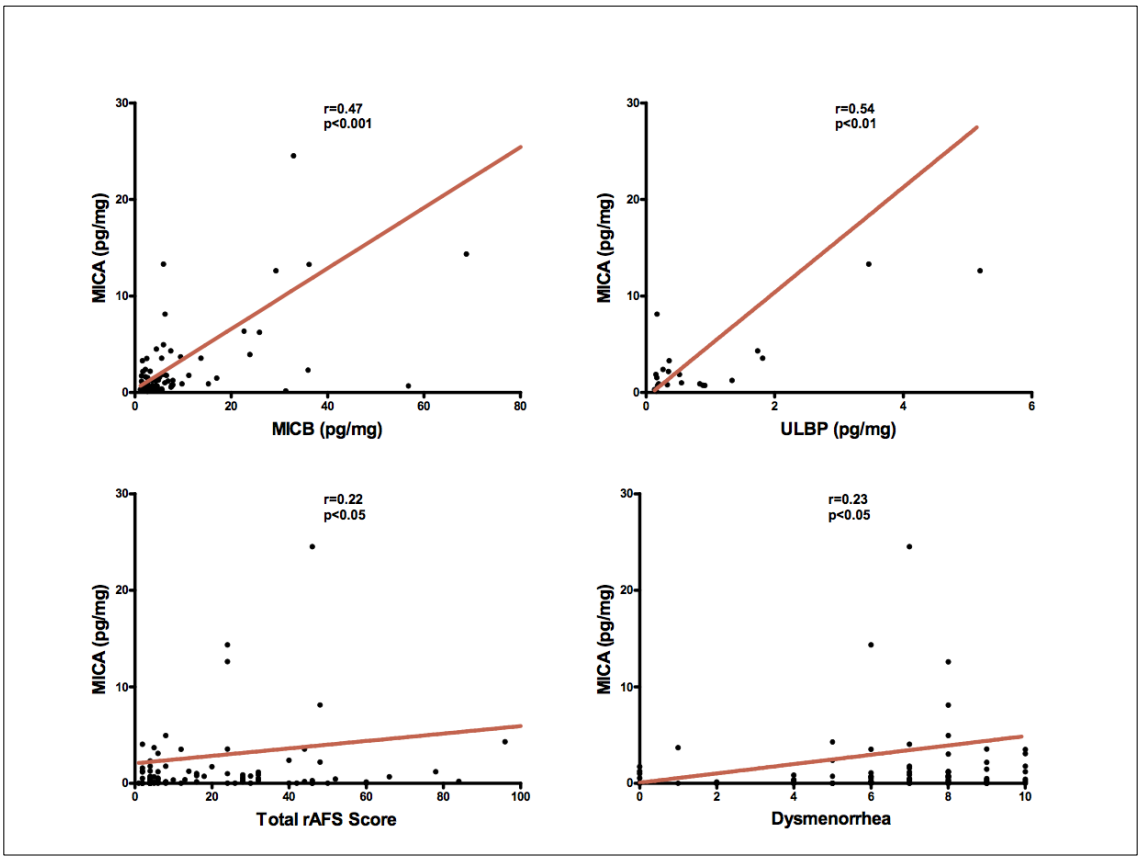
<u>Table S3</u>. Correlation analysis of peritoneal fluid MICA, MICB and ULBP-2 levels and clinical data in women with endometriosis.

Measurements	MICA		MICB		ULBP-2	
	Spearman Rank Correlation Coefficient (r)	p	Spearman Rank Correlation Coefficient (r)	p	Spearman Rank Correlation Coefficient (r)	p
Dysmenorrhea	0.232	0.029	0.071	0.507	0.125	0.245
Deep dyspareunia	0,157	0.151	0.089	0.416	-0.007	0.949
Non cyclic chronic pelvic pain	0.086	0.413	-0.002	0.983	-0.076	0.471
Gastrointestinal symptoms	0.098	0.368	0.036	0.740	0.057	0.603
Lower urinary tract symptoms	-0.125	0.250	0.027	0.803	0.017	0.875
Total rAFS score	0.221	0.031	0.088	0.399	0.197	0.055
Implants rAFS score	0.188	0.068	0.010	0.920	0.197	0.055
Adhesions rAFS score	0.221	0.031	0.103	0.319	0.217	0.034
Total number of DIE lesions *	-0.060	0.718	0.085	0.606	0.210	0.200
MICA	N.A.	N.A.	0.466	0.000	0.540	0.009
MICB	0.466	0.000	N.A	N.A	0.322	0.101
ULBP-2	0.540	0.009	0.322	0.101	N.A	N.A

Note: Pain intensity was evaluated preoperatively using a previously validated 10-cm VAS scale. (113) rAFS: according to The Revised American Fertility Society classification of endometriosis (107).

* Only in DIE patients.

<u>Figure S2</u>. Correlation analysis of PF MICA levels with MICB, ULBP-2 and with total rAFS score and dysmenorrhea score. Non-parametric Spearman's correlation tests was used to assess correlations.



11. ARTICLES

Article 1

Dysregulation of the ADAM/Notch signalling pathway in endometriosis: from oxidative stress to fibrosis

Molecular Human Reproduction, pp. 1–12, 2017
doi:10.1093/molehr/gax028

molecular human reproduction

ORIGINAL ARTICLE

Dysregulation of the ADAM17/Notch signalling pathways in endometriosis: from oxidative stress to fibrosis

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Submitted on February 14, 2017; resubmitted on April 10, 2017; editorial decision on April 19, 2017; accepted on April 28, 2017

STUDY QUESTION: Is oxidative stress associated with the A disintegrin and metalloproteases (ADAM) metallopeptidase domain 17 (ADAM17)/Notch signalling pathway and fibrosis in the development of endometriosis?

SUMMARY ANSWER: Oxidative stress is correlated with hyperactivation of the ADAM17/Notch signalling pathway and a consequent increase in fibrosis in patients with endometriosis.

WHAT IS KNOWN ALREADY: It is nowadays accepted that oxidative stress plays an important role in the onset and progression of endometriosis. Oxidative stress is able to induce the synthesis of some members of the 'ADAM' family, such as ADAM17. ADAM17/Notch signalling is dysregulated in other profibrotic and inflammatory diseases.

STUDY DESIGN, SIZE, DURATION: This was a prospective laboratory study conducted in a tertiary-care university hospital between January 2011 and April 2013. We investigated non-pregnant, younger than 42-year-old patients ($n = 202$) during surgery for a benign gynaecological condition.

PARTICIPANTS/MATERIALS, SETTING, METHODS: After complete surgical exploration of the abdominopelvic cavity, 121 women with histologically proven endometriosis and 81 endometriosis-free control women were enrolled. Peritoneal fluid (PF) samples were obtained from all the study participants during surgery in order to detect advanced oxidation protein products (AOPPs) and metalloproteinase activity of ADAM17. Stromal cells from endometrial specimens ($n = 8$) were obtained from endometrium of control patients (*Cs*), and from eutopic (*Es*) and ectopic (*Ps*) endometrium of patients with deep infiltrating endometriosis (DIE) ($n = 8$). ADAM17, Notch and the fibrosis markers α-smooth muscle actin (α-SMA) and type-I collagen were assessed using immunoblotting in all the endometrial samples obtained. Additionally, fibrosis was assessed after using Notch cleavage inhibitors (DAPT and FLI-06). Notch and fibrosis were also evaluated after stimulation of stromal endometrial cells with ADAM17 purified protein, increasing concentrations of H_2O_2 and primary cell culture supernatants.

MAIN RESULTS AND THE ROLE OF CHANCE: Patients with DIE presented higher PF AOPP and ADAM17 protein levels than controls ($P < 0.01$ and $P < 0.05$, respectively). In addition, these two markers were positively correlated ($r = 0.614$; $P < 0.001$). At the cellular level, ADAM17 activity was increased in *Es* and *Ps* compared to *Cs* ($P < 0.001$ and $P < 0.01$, respectively). Furthermore, *Ps* presented hyperactivation of Notch signalling ($P < 0.05$) and augmentation of fibrosis markers ($P = 0.009$ for α-SMA and $P = 0.015$ for type-I collagen) compared to controls. The use of DAPT and FLI-06 reduced both fibrosis markers in *Ps* but not in *Cs*. Stimulation with ADAM17, H_2O_2 and *Ps* supernatant culture significantly increased Notch and fibrosis in both *Ps* and *Cs*.

LARGE SCALE DATA: N/A.

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LIMITATIONS REASONS FOR CAUTION: The control group consisted of women who underwent surgery for benign gynaecological conditions, which could lead to biases because some of these conditions may cause alterations in oxidative stress and the ADAM17/Notch pathways. The small sample size of endometrial biopsies used for each group of patients ($n = 8$) is a limitation of the study, and results should be interpreted with caution.

WIDER IMPLICATIONS OF THE FINDINGS: We propose a novel pathway in endometriosis pathogenesis that correlates oxidative stress, hyperactivation of ADAM17/Notch signalling and a consequent increase in fibrosis. This study suggests that Notch signalling plays a key role in the fibrotic processes that take place in ectopic lesions of patients with DIE, as already observed in other pro-fibrotic diseases.

STUDY FUNDING AND COMPETING INTEREST(S): This work was supported by grants from University Paris Descartes, INSERM and Fundación Alfonso Martín Escudero. The authors have no competing interests to declare.

Key words: endometriosis / ADAM17 / Notch / oxidative stress / fibrosis

Introduction

Endometriosis is a benign gynaecological disease characterized by the presence of endometrial tissue outside the uterine cavity (Giudice and Kao, 2004). Previous reports have shown that this disease may affect up to 10–15% of women of reproductive age, representing a major public health issue (de Ziegler *et al.*, 2010). Although the pathogenesis of this disorder is not completely clear (Carmona *et al.*, 2013; Luisi and Santulli, 2013), it is known that endometriosis is a complex chronic inflammatory condition (Bulun, 2009) associated with an overproduction of prostaglandins, metalloproteases, cytokines and chemokines (Santulli *et al.*, 2014). These inflammatory agents produce a self-supporting loop that sustains and increases the development of the disease (Yuge *et al.*, 2007).

Recent findings have demonstrated that oxidative stress is part of this inflammatory process and plays a pivotal role in the onset and progression of different forms of endometriosis such as ovarian endometrioma (OMA) and deep infiltrating endometriosis (DIE) (Ngo *et al.*, 2009; Santulli *et al.*, 2015). It has been shown that oxidative stress is able to induce the synthesis of some members of the 'A disintegrin and metalloproteases' (ADAM) family, such as ADAM metallopeptidase domain 17 (ADAM17) (also called TACE, for tumour-necrosis-factor alpha converting enzyme) (Zhang *et al.*, 2001). ADAM family members are proteolytically active over a large variety of target proteins with immunological relevance (Miller *et al.*, 2013), such as Notch for ADAM17. Notch proteins are transmembrane receptors that regulate many developmental processes such as proliferation, differentiation and cell apoptosis (Hori *et al.*, 2013). Among these functions, Notch may promote fibroblast proliferation, and its dysfunctions have been related to a wide spectrum of diseases, including skin, lung, kidney and cardiac fibrosis (Kavian *et al.*, 2012a,b).

However, so far, there are no data evaluating the role of the ADAM17/Notch signalling pathway in endometriosis.

The aim of the present study was to find a pathway that associates chronic inflammation, stromal cell dysfunction and fibrosis with the pathogenesis of endometriosis. We, therefore, assessed the relationship between oxidative stress, ADAM17 activity, Notch cleavage and fibrosis in patients with and without endometriosis.

Material and Methods

Patients

The local Ethics Committee (CCPPRB: Comité Consultatif de Protection des Personnes dans la Recherche Biomédicale) of our institution approved the study protocol and all study participants gave informed written consent for enrolment in the study protocol.

From January 2011 to April 2013, a consecutive series of 202 <42-years-old, non-pregnant women who underwent surgery (operative laparoscopy or laparotomy) for a benign gynaecological disorder in our centre were recruited for the study. Clinical and biological data were prospectively collected for all the patients. Women with cancer or who were pregnant and those who did not give their informed consent were excluded from the study. The women were classified into two groups depending on surgical findings (Chapron *et al.*, 2011a,b): the endometriosis group consisted of subjects with histologically proven endometriosis, while patients in the control group did not show any macroscopic sign of the disease after a meticulous exploration of the abdominal cavity during the surgical procedure.

Endometriosis was staged and scored (total, implant and adhesion scores) according to the revised American Fertility Society Classification (1985). In addition, patients with endometriosis were also staged according to the endometriosis phenotype. Based on histological findings, endometriotic lesions were classified into three groups: peritoneal superficial endometriosis (SUP), OMA and DIE. As these three types of lesions may coexist (Somigliana *et al.*, 2007), patients with endometriosis were classified according to the most severe finding. Endometriotic lesions are usually ranked from the least severe to the most severe in SUP, OMA and DIE (Chapron *et al.*, 2009).

The study analysis used a prospectively managed database. For each patient, personal history data were obtained during face-to-face interviews, which were conducted by the surgeon the month before surgery. A highly structured previously published questionnaire was used for all patients (Chapron *et al.*, 2010a,b). The following items were recorded: age, parity, gravidity, height, weight, BMI and past history of hormonal and/or surgical treatment for endometriosis. The use of antigonadotrophic oral contraceptives (OCs) was recorded in each group and was defined as the use of an OC for at least 6 months before surgery (Chapron *et al.*, 2011a,b; Santulli *et al.*, 2014).

Collection of peritoneal fluid samples

Peritoneal fluid (PF) was taken during surgery from all the patients included in the study ($n = 202$). The samples were centrifuged at 800*g* for 10 min at 4°C and supernatants were collected. Aliquots of the samples were stored at −80°C until analysis. PF protein concentration was measured in all the samples using the spectroscopic Bradford protein assay method (Bradford, 1976).

Advanced oxidation protein products in PF

Advanced oxidation protein products (AOPPs) were measured as previously described (Witko-Sarsat *et al.*, 1998). Briefly, 200 µl of PF diluted 1:5 in phosphate-buffered saline (PBS) were placed into each well of a 96-well

microtitre plate. Afterwards, 20 µl of acetic acid were added into each well. For the standards, 10 ml of 1.16 M potassium iodide (Sigma, St Louis, MO, USA) were added to 200 ml of chloramine-T solution (0–100 mmol/l) (Sigma, St Louis, MO, USA) in each well and then 20 ml of acetic acid was added. The absorbance of the reaction mixture was immediately read at 340 nm against a blank consisting of 200 ml of PBS, 10 ml of 1.16 M potassium iodine and 20 ml of acetic acid. In order to avoid biases due to PF concentrations or dilutions, a ratio between AOPP (µmol/l) and protein concentrations (mg/ml) was calculated for each peritoneal sample individually (values are expressed in nmol/mg).

Metalloproteinase activity of ADAM17 protein in PF

PFs were prepared as described previously (Santulli *et al.*, 2015). We used the Mca-PLAQAV-Dpa-RSSSR-NH2 Fluorogenic Peptide Substrate III (R&D Systems, Inc., Minneapolis, MN, USA, Catalogue # ES003) for the assay of ADAM17 activity. Fluorogenic peptide substrate III was used to measure the activities of peptidases that are capable of cleaving an amide bond between the fluorescent group and the quencher group, causing an increase in fluorescence. The peptide sequence is derived from the pro-tumour necrosis factor-α (pro-TNF-α). This peptide acts as substrate for TNF-α converting enzyme (TACE/ADAM17). Fluorogenic peptide substrate III (50 µl) was directly added to 50 µl of PF for 1 h at 37°C. Fluorescence was recorded after 60 min on a spectrofluorimeter (Fusion; Packard, USA) at 320 nm and 405 nm as excitation and emission wavelengths, respectively. The fluorescence intensity strongly reflects the ADAM17 enzyme activity (Caescu *et al.*, 2009).

In order to avoid biases owing to PF concentrations or dilutions, a ratio between the ADAM17 fluorescence result (pg/ml) and the protein concentration (mg/ml) was calculated for each PF sample individually (values are expressed in pg/mg).

Tissue collection, cell isolation and culture

Endometrial biopsy specimens were collected from control patients ($n = 8$) without any macroscopic endometriotic lesion after a thorough surgical examination of the abdominopelvic cavity. Indications for surgery in controls were the following: infertility, fibroids or non-endometriotic ovarian cysts. All samples were histologically characterized for patients and controls (Noyes *et al.*, 1975). Both eutopic and ectopic endometrium were obtained from patients with endometriosis ($n = 8$). In the endometriosis group the ectopic implant consisted in low rectal endometriosis nodules, defined by full-thickness invasion of the muscular layer of the rectum (Chapron *et al.*, 2010a,b).

Primary endometrial and deep endometriotic cell cultures were prepared from biopsies as described previously (Ngo *et al.*, 2009). Biopsy specimens were rinsed and minced into small pieces then digested with 5% dispase and collagenase (2 mg/ml, Gibco Invitrogen, Cergy Pontoise, France) for 1 h at 37°C and separated using serial filtration. Red blood cells were removed by hypotonic lysis (using 0.15 M NH_4Cl, 1 mM $KHCO_3$, 0.1 mM Na_2 EDTA). Debris was removed using sieves with 100 µm apertures; epithelial cells were retained on sieves with 40 µm apertures while stromal cells remained in the filtrate. Stromal cells were plated onto Primaria flasks (Becton Dickinson Labware, Le Pont de Claix, France) and cultured in DMEM (Gibco Invitrogen, Cergy Pontoise, France) with 10% foetal calf serum. Two populations of cells were obtained from each patient with DIE: eutopic endometrial stromal cells (*Es*), and DIE stromal cells (*Ps*). For each control we used one cell population of eutopic endometrial stromal cells (*Cs*).

The purity of stromal and epithelial cell suspensions was assessed by staining with 1:100 FITC-labelled anti-cytokeratin and 1:100 Cy3-labelled anti-vimentin antibodies (Sigma-Aldrich, St Louis, MI, USA). Fluorescence was analysed using an Olympus fluorescent microscope (Hamburg, Germany) and images were captured using the Cell Imaging station (Olympus). Both populations were negative for CD3 (T cells), CD45 (leucocytes) and CD11b (monocytes and granulocytes) staining. All the experiments were performed on primary cultures of each cell population, and the various tests were performed in triplicate. Primary cultures were obtained between 7 and 14 days after collecting the samples. Oestrogen and progesterone were undetectable in cell culture supernatants as determined by an immunodiagnostic system (Advia Centaur XP, Siemens Health Care Diagnostics, Saint-Denis, France).

Immunoblotting measurement of ADAM17, activated notch and α-smooth muscle actin in endometrial cells

Equal amounts of proteins (40 µg) were loaded and separated by 10% sodium dodecyl sulphate (SDS) polyacrylamide gel electrophoresis. Transfer and blocking were performed as follows. Polyacrylamide membranes were saturated with 5% skimmed milk for 1 h at room temperature and incubated overnight at 4°C with Cleaved Notch-1 (Val 1744) Antibody (Cell Signaling Technology, Danvers, MA, USA) and Human ADAM17 Antibody (R&D Systems, Inc., Minneapolis, MN, USA), respectively. The membranes were then washed and specific antibodies were detected using a 1:1000 dilution of horse-radish peroxidase (HRP)-conjugated goat anti-rabbit IgG Abs (Dako, Denmark) and visualized by an Enhanced Chemiluminescence system (Advansta, CA, USA). After film exposure, the membranes were washed three times for 10 min each in TBST (TBS-Tween 20). Membranes were incubated for 30 min at 50°C in stripping buffer (100 mM β-mercaptoethanol, 2% SDS, 62.5 mM Tris–HCl, pH 6.7). Afterwards, they were washed three more times for 10 min each in TBST. Membranes were saturated with 5% skimmed milk for 1 h at room temperature and then incubated with a 1:50 000 dilution of a peroxidase-labelled monoclonal anti β-actin antibody (Sigma) for 1 h at room temperature and visualized by an Enhanced Chemiluminescence system (Advansta, Menlo Park, CA, USA). Optical densities (ODs) were measured using Multigauge Software (Fujifilm, Tokyo, Japan). A ratio between ADAM17 OD or Notch OD and β-actin OD was calculated in each case (arbitrary units are presented).

Similarly, the expression of α-smooth muscle actin (α-SMA) was assessed using western blot with an anti-mouse α-SMA antibody (clone 1A4; Sigma). Specific antibodies were detected using a HRP-conjugated goat anti-rabbit IgG antibody (Dako, Denmark) and visualized by an enhanced Chemiluminescence system (Advansta, Menlo Park, CA, USA). Expression was calculated as a ratio between α-SMA OD divided by β-actin (Sigma) OD (arbitrary units are presented).

Effect of Notch cleavage inhibition by DAPT and FLI-06 on α-SMA expression and type-I collagen production in endometrial cells

Endometrial stromal cells from ectopic nodules of DIE patients (*Ps* cells) and endometrial stromal cells from control patients (*Cs* cells) were incubated with either 100 µM DAPT (*N*-*S*-phenyl-glycine-*t*-butyl ester) (Sigma, St Louis, MI, USA) or 2.3µM FLI-06 (Selleckchem, Houston, TX, USA), both being γ-secretase inhibitors.

Cleaved Notch and α-SMA expression in endometrial cells after stimulation with ADAM17 purified protein, increasing concentrations of H_2O_2 and culture supernatants

Additionally, *Cs* and *Ps* cells were also incubated with H_2O_2 at increasing concentrations, ranging from 0 to 40 µM, or stimulated with *Cs* and *Ps* cell culture supernatants or incubated with ADAM17 purified protein (0.01 µg/ml) (R&D system northeast MN, USA). For each condition, cells were cultured for 24 h at 37°C.

Collagen content assay

After 24 h of treatment with DATP (100 µM) and FLI-06 (2.3 µM), the samples were stained with 1% Picro-sirius red for 1 h and cells were then washed three times with acetic acid (0.5% in H_2O). Staining was eluted using sodium hydroxide and quantified at 540 nm as OD. Type-I collagen was evaluated as arbitrary units per million cells.

Statistical analysis

Data were analysed using GraphPad Prism five software (GraphPad Software, Inc., San Diego, CA, USA). The Student's *t*-test was used for quantitative variables and the Pearson's Chi-square or Fisher's exact test for qualitative variables, as appropriate. Considering the non-Gaussian distribution of our biological parameters, statistical analysis was performed between two groups using non-parametrical statistical tests. All quantitative data are expressed as the median (range). Analyses between two groups were performed using the Mann–Whitney non-parametric *U*-test. When the analysis included more than two groups, the Kruskal–Wallis test was used. The *post hoc* tests were performed using Dunn's multiple comparison test. The non-parametric Spearman correlation test was used to assess correlations.

Comparison of α-SMA and collagen in both groups of cells was performed using a non-parametric test for matched samples (Wilcoxon matched pairs test).

P-values < 0.05 were considered significant.

Results

Patients and controls

One hundred and twenty-one women with endometriosis and 81 disease-free women were included in the study. The patients' clinical and surgical characteristics are shown in Table I.

Following the endometriosis surgical classification (Chapron *et al.*, 2010a,b) and based on the location of the worst lesion presented, the 121 histologically proven endometriotic patients were classified as follows: 41 (33.9%) SUP, 32 (26.4%) OMA (right 11; left 14; bilateral 7) and 48 (39.7%) DIE.

All surgery indications in the 81 endometriosis-free women were for the following benign gynaecological conditions: uterine fibroids (29 patients, 35.8%), non-endometriotic benign ovarian cysts (21 patients, 25.9%), tubal infertility (15 patients, 18.6%), pelvic pain (7 patients, 8.6%) and other indications (9 patients, 11.1%).

No differences were detected in age, gravidity, parity and infertility between the endometriosis group and control group. The BMI was significantly lower in endometriotic patients than controls ($P = 0.001$). The percentage of patients with preoperative hormonal treatment was similar in the two groups (Table I).

Table I Baseline characteristics of participants in a study of signalling pathways in endometriosis.

Patient characteristics	Endometriosis (*N* = 121)	Controls (*N* = 81)	*P*
Age (years)[a]	30.8 ± 5.1	31.7 ± 5.36	0.267[t]
Height (cm)[a]	167.5 ± 6.1	164.1 ± 6.0	<0.001[t]
Weight (kg)[a]	59.5 ± 8.1	61.5 ± 9.9	0.162[t]
BMI (kg/m^2)[a]	21.2 ± 2.5	22.8 ± 3.4	0.001[t]
Parity[a]	0.2 ± 0.5	0.4 ± 0.7	0.093[t]
Gravidity[a]	0.4 ± 0.7	0.6 ± 1.0	0.123[t]
Preoperative hormonal treatment (*n*, %)	48 (40.0%)	33 (40.1%)	0.887[k]
Previous treatment for endometriosis			
Hormonal treatment (*n*, %)*	68 (56.2%)	NA	
Previous surgery (*n*, %)	19 (15.7%)	NA	
Previous endometrioma's surgery (*n*, %)	11 (9.1%)	NA	
rAFS classification			
Mean implants score rAFS[a,b]	11.3 ± 11.2	NA	
Mean adhesions score rAFS[a,b]	9.6 ± 16.3	NA	
Mean total score rAFS[a,b]	21.2 ± 23.1	NA	
rAFS stage (*n*, %)[b]		NA	
I	37(30.6%)		
II	16 (13.2%)		
III	26 (21.5%)		
IV	42 (34.7%)		
Surgical classification			
SUP (*n*, %)	41 (33.9%)	NA	
Endometrioma (*n*,%)	32 (26.4%)	NA	
Endometrioma laterality (*n*, %)		NA	
Bilateral	7/32 (21.9%)		
Right	11/32 (34.4%)		
Left	14/32 (43.7%)		
DIE lesions (*n*, %)[c]	48 (39.7%)	NA	

NA, not applicable; DIE, deep infiltrating endometriosis; SUP, superficial endometriosis; rAFS, revised American Fertility Society.
[a]Data are presented as mean ± SD.
[b]Score according to the rAFS Classification (1985).
[c]According to a previously published surgical classification for DIE (Chapron *et al.*, 2006).
[t]Student's *t*-test.
[k]Pearson's chi-square test.
*<5% missing data.

AOPP levels are increased in endometriosis and correlate with ADAM17

In this work, AOPP and ADAM17 were measured in PF from all the study population ($n = 202$). The levels of AOPP were significantly increased in endometriotic patients compared to controls (median, 1.73 nmol/mg; range, 0.43–411.9 versus median, 1.25 nmol/mg; range, 0.27–18.26; $P < 0.001$) (Fig. 1A). According to the surgical classification, AOPP levels were different among groups ($P < 0.01$). A *post*

hoc test showed a significant increase in PF AOPP levels in DIE patients (which represent the most severe forms of the disease) as compared to controls (median, 2.22 pg/mg; range, 0.63–411.9 versus median, 1.30 pg/mg; range, 0.28–18.26; $P < 0.01$) (Fig. 1B).

We found a significantly higher activity of ADAM17 metalloprotease in endometriosis patients than in women without the disease (median, 0.28 pg/mg; range, 0.06–469.6 versus median, 0.20 pg/mg; range, 0.07–52.7; $P = 0.019$) (Fig. 1C). According to the surgical classification, ADAM17 levels were significantly different among groups ($P < 0.05$). The *post hoc* test revealed increased PF ADAM17 levels in DIE patients as compared to controls (median, 0.34 pg/mg; range, 0.06–469.6 versus median, 0.20 pg/mg; range, 0.07–52.7; $P < 0.05$) (Fig. 1D).

In addition, a significant biological correlation was found between the AOPP PF levels of patients with endometriosis and ADAM17 activity ($r = 0.614$; $P < 0.001$) (Fig. 1E).

In order to rule out any bias concerning the effect of preoperative hormonal treatment, we compared AOPP and ADAM17 levels in patients who used hormonal treatment with patients who did not. We failed to show any differences in any of the biomarkers according to the use of OC ($P = 0.934$ and $P = 0.406$ for AOPP and ADAM17, respectively).

ADAM17 activity is up-regulated in stromal endometrial cells from die patients

ADAM17 protein level was assessed by western blot in all the different primary cell cultures derived from *Cs* of control patients and *Es* and *Ps* endometrium of DIE patients (Fig. 2A).

ADAM17 expression in *Cs*, *Es* and *Ps* cells was significantly different among groups (*Cs* mean OD, 0.11; SEM ± 0.02 versus *Es* mean, 0.63; SEM ± 0.10 versus *Ps* mean, 0.53; SEM ± 0.07; $P < 0.001$). The *post hoc* test showed increased ADAM17 activity in *Es* and *Ps* cells compared to *Cs* cells ($P < 0.001$ and $P < 0.01$, respectively) (Fig. 2A).

Notch is hyperactivated in patients with endometriosis

Notch activation was evaluated using western blot by measuring the amounts of Notch intracellular domains (NICDs) contained in the different primary cell cultures derived from *Cs*, *Es* and *Ps* cells. Higher amounts of NICD were shown in stromal endometrial cells from DIE patients in comparison with disease-free patients (*Cs* mean OD, 0.17; SEM ± 0.03 versus *Es* mean, 0.34; SEM ± 0.10 versus *Ps* mean, 0.39; SEM ± 0.08; $P = 0.040$). A *post hoc* test revealed a significant increase of Notch signalling in *Ps* compared to *Cs* cells ($P < 0.05$). The differences observed between *Cs* cells and *Es* cells did not reach statistical significance (Fig. 2B).

Increased Notch activity in endometriosis patients leads to a major activation of fibroblasts

Basal levels of α-SMA and type-I collagen were significantly increased in *Ps* compared to *Cs* cells (*Ps* mean OD for α-SMA, 0.80; SEM ± 0.14 versus *Cs* mean, 0.54; SEM ± 0.10; $P = 0.009$; *Ps* mean OD for type-I collagen, 0.53; SEM ± 0.07 versus *Cs* mean, 0.34; SEM ± 0.03; $P = 0.015$) (Fig. 3).

Notch cleavage inhibition only reduces fibrosis in ectopic stromal endometrial cells (*Ps*) from die but not in eutopic stromal endometrial cells (*Cs*) from controls

After treatment with DAPT and FLI-06 (both γ-secretase inhibitors that prevent Notch cleavage and NICD release) *Ps* cells presented a significant reduction of α-SMA (*Ps* untreated mean OD, 0.80; SEM ± 0.14 versus *Ps* DAPT mean, 0.45; SEM ± 0.14; $P = 0.006$; *Ps* untreated versus *Ps* FLI-06 mean, 0.47; SEM ± 0.17; $P = 0.031$) (Fig. 3A and B) and type-I collagen expression (*Ps* untreated mean OD, 0.53; SEM ± 0.07 versus *Ps* DAPT mean, 0.39; SEM ± 0.02; $P = 0.048$; *Ps* untreated versus *Ps* FLI-06 mean, 0.37; SEM ± 0.06; $P = 0.039$) (Fig. 3C).

Notwithstanding, no differences were observed in α-SMA levels when *Cs* cells with normal amounts of NICD were treated with DAPT and FLI-06, (*Cs* untreated mean OD, 0.54; SEM ± 0.10 versus *Cs* DAPT mean, 0.48; SEM ± 0.07; $P = 0.276$; *Cs* untreated versus *Cs* FLI-06 mean, 0.45; SEM ± 0.11; $P = 0.149$) (Fig. 3A and B) or in type-I collagen levels (*Cs* untreated mean OD, 0.34; SEM ± 0.03 versus *Cs* DAPT mean, 0.33; SEM ± 0.04; $P = 0.999$; *Cs* untreated versus *Cs* FLI-06 mean, 0.32; SEM ± 0.04; $P = 0.808$) (Fig. 3C).

Cleaved Notch and α-SMA expression after stimulation of ectopic stromal endometrial cells (*Ps*) and eutopic stromal endometrial cells of controls (*Cs*) with adam17 purified protein, increasing concentrations of H_2O_2 and culture supernatants

Stimulation with ADAM17 purified protein generated a significant increase in cleaved Notch both in *Cs* (*Cs* untreated mean OD, 0.20; SEM ± 0.06 versus *Cs* + ADAM17 mean, 0.56; SEM ± 0.04; $P = 0.013$) and *Ps* cells (*Ps* untreated mean OD, 0.60; SEM ± 0.17 versus *Ps* + ADAM17 mean, 1.38; SEM ± 0.21; $P = 0.001$) (Fig. 4A), It is noteworthy that the increase was much higher in *Ps* cells. Similarly, ADAM17 stimulation augmented α-SMA expression in *Cs* (*Cs* untreated mean OD, 0.42; SEM ± 0.02 versus *Cs* + ADAM17 mean, 0.75; SEM ± 0.08; $P = 0.016$) and *Ps* cells (*Ps* untreated mean OD, 0.81; SEM ± 0.09 versus *Ps* + ADAM17 mean, 1.60; SEM ± 0.13; $P = 0.002$) (Fig. 4B).

Furthermore, when stromal endometrial cells were incubated with increasing concentrations of H_2O_2, both *Cs* and *Ps* cells showed a progressive increase in cleaved Notch levels ($P < 0.05$ and $P < 0.01$ for *Cs* and *Ps* cells, respectively) (Fig. 5A) and in α-SMA expression ($P < 0.05$ and $P < 0.01$) (Fig. 5B).

In order to evaluate if *Cs* and *Ps* cell culture supernatants were sufficient to induce an increase of Notch and α-SMA, both types of cells were incubated with their counterpart supernatant and vice-versa. Rising Notch concentrations were observed when *Cs* and *Ps* cells were treated with *Ps* supernatants. The *post hoc* test showed a significant increase of Notch expression in *Cs* cells treated with *Ps* supernatant (*Cs* untreated mean OD, 0.26; SEM ± 0.08 versus *Cs* + *Ps* supernatant mean, 0.81; SEM ± 0.24; $P = 0.036$) (Fig. 6A), and in *Ps* cells treated with *Ps* supernatant compared to their basal untreated cells (*Ps* untreated mean OD, 0.69; SEM ± 0.07 versus *Ps* + *Ps* supernatant mean, 1.75; SEM ± 0.05; $P = 0.003$) (Fig. 6B). No differences in Notch

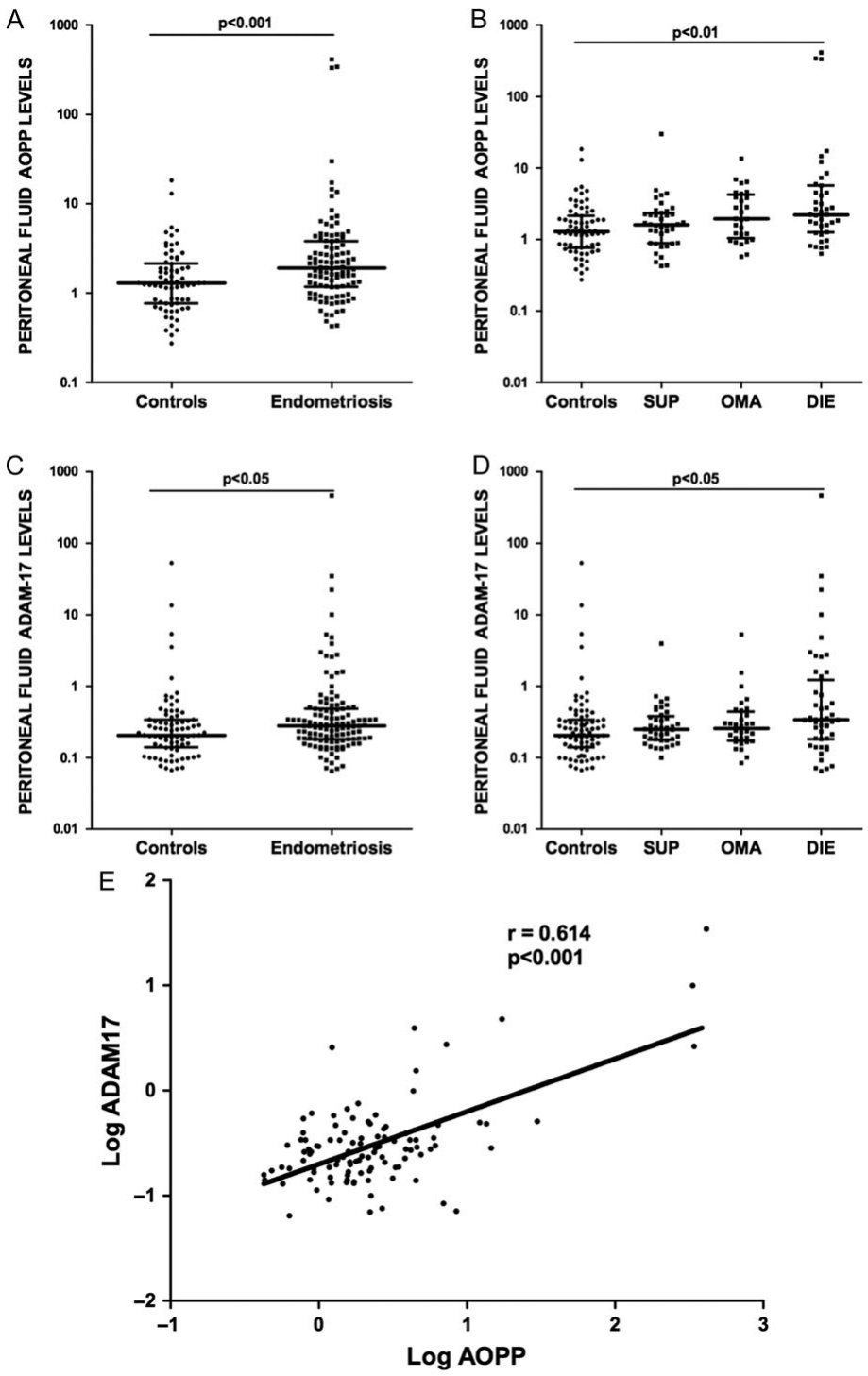

Figure 1 AOPPs and ADAM17 levels in patients with endometriosis and controls. (**A**) AOPP measured in PF by ELISA in patients with endometriosis ($n = 121$) and in controls ($n = 81$) after exploration of the abdominopelvic cavity. (**B**) AOPP levels measured by ELISA according to the surgical classification of endometriosis [controls ($n = 81$); SUP ($n = 41$); OMA ($n = 32$) and DIE ($n = 48$)]. (**C**) ADAM17 levels measured by ELISA in PF of endometriosis patients and controls. (**D**) ADAM17 levels according to the surgical classification of endometriosis. (**E**) Correlation analysis of PF AOPP and ADAM17 proteinase levels in 121 patients with endometriosis. The logarithmic transformation of PF AOPP and ADAM17 levels is depicted. Statistical analyses were performed using the Mann–Whitney non-parametric *U*-test for figures A and C, and the Kruskal–Wallis test for figures B and D. The results of pairwise comparisons using Dunn's Multiple Comparison test are presented (B, D). The non-parametric Spearman's correlation test was used to assess correlations (Fig. E). ADAM17 and AOPP values are represented on a logarithmic scale as a scatter dot plot. ADAM17 values are expressed in pg/mg. AOPP values are expressed in nmol/mg. The medians with their interquartile range are reported. PF, peritoneal fluid; AOPP, advanced oxidation protein products. DIE, deep infiltrating endometriosis; SUP, superficial endometriosis; OMA, ovarian endometrioma; ADAM17, ADAM metallopeptidase domain 17.

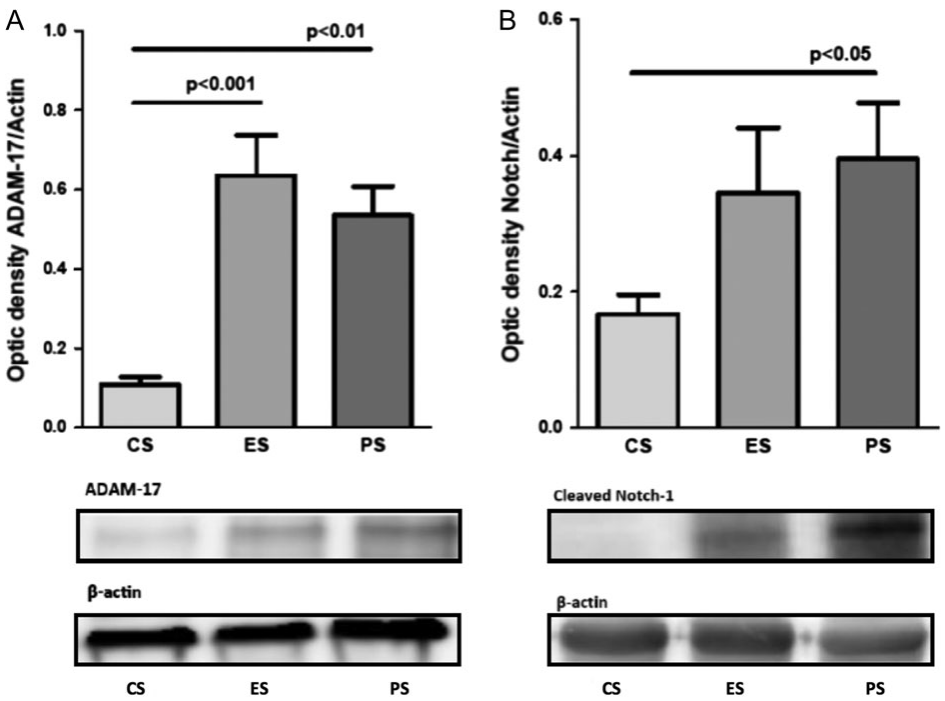

Figure 2 ADAM17 and Notch-1 intracellular domain (NICD) levels in stromal endometrial cells of control women and patients with DIE.
Differences in ADAM17 activity (**A**) and NICD levels (**B**) among eutopic stromal endometrial cells (*Cs*) of control patients ($n = 8$), and stromal cells of eutopic (*Es*) ($n = 8$) and ectopic (*Ps*) endometrium of DIE patients ($n = 8$) assessed by western blot of cell lysates. Statistical analyses were performed using the Kruskall–Wallis test. Dunn's Multiple Comparison test was used for *post hoc* analysis. The mean OD ratio for ADAM17/β-actin and Notch/β-actin was calculated for each endometriotic cell type and compared with control endometrial cells. Error bars represent SEM. Arbitrary units are reported. OD, optical density; NICDs, Notch-1 intracellular domains.

were observed when *Cs* and *Ps* cells were treated with *Cs* supernatant. Accordingly, α-SMA assessment revealed differences in its levels when both *Cs* and *Ps* cells were incubated with *Ps* supernatants. The *post hoc* test analysis revealed a significant increase in α-SMA expression in *Cs* cells treated with *Ps* supernatant (*Cs* untreated mean OD, 0.44; SEM ± 0.06 versus *Cs* + *Ps* supernatant mean, 0.84; SEM ± 0.11; $P = 0.028$) (Fig. 6C), and in *Ps* cells incubated with their own *Ps* supernatant (*Ps* untreated mean OD, 0.69; SEM ± 0.12 versus *Ps* + *Ps* supernatant mean, 2.75; SEM ± 0.36; $P = 0.005$) (Fig. 6D). Notwithstanding, when *Cs* and *Ps* cells were treated with *Cs* supernatant, no significant increase in α-SMA expression was observed.

Discussion

To the best of our knowledge, this is the first report that associates oxidative stress with the ADAM17/Notch signalling pathway and fibrosis in endometriosis patients (Fig. 7). The aim of the present study was to find a pathway that linked chronic inflammation with the stromal cell dysfunction and fibrosis that occur in endometriosis.

It is nowadays accepted that endometriosis is an inflammatory disease (de Ziegler *et al.*, 2010). According to previous publications (Ngo *et al.*, 2009; Leconte *et al.*, 2011), oxidative stress is altered in endometriotic patients, and plays a crucial role in the onset and progression of the disorder. In this study, the levels of AOPP, which reflect the intensity of oxidative stress (Witko-Sarsat *et al.*, 1996), were measured in PF and found to be significantly elevated in women with endometriosis, especially in patients with DIE, confirming previous observations (Santulli *et al.*, 2015). A significant positive correlation was found between AOPP PF levels of patients with endometriosis and ADAM17 proteinase levels. These levels were also significantly increased in patients with endometriosis compared to controls. According to the surgical classification, AOPP levels (Santulli *et al.*, 2015), and the highest ADAM17 levels were found in the PF of patients with DIE, the most severe form of endometriosis. At cellular level, ADAM17 activity was significantly increased in stromal cells of ectopic (*Ps*) and eutopic (*Es*) endometrium of DIE patients compared to eutopic stromal endometrial cells (*Cs*) of control patients.

ADAM17 is a 70-kDa enzyme that belongs to the ADAM protein family of disintergrins and metalloproteases. ADAM17 expression is increased during inflammatory processes and especially upon reactive oxygen species (ROS) exposure (Wang *et al.*, 2009; Kavian *et al.*, 2010; Scott *et al.*, 2011). ADAM17 is understood to be involved in the processing of TNF-α on the surface of the cell. Recently, ADAM17 has been shown to induce the cleavage of NKG2D ligands from the cell surface, thus increasing the concentration of free soluble ligands (Chitadze *et al.*, 2013), the levels of which are increased in PF of patients with DIE (Gonzalez-Foruria *et al.*, 2015). Previous *in vitro* experiments have provided evidence that ADAM17 also plays a prominent role in the Notch signalling pathway, during the proteolytic release of the NICD (from the Notch-1 receptor) that occurs following ligand binding (Kavian *et al.*, 2012a,b).

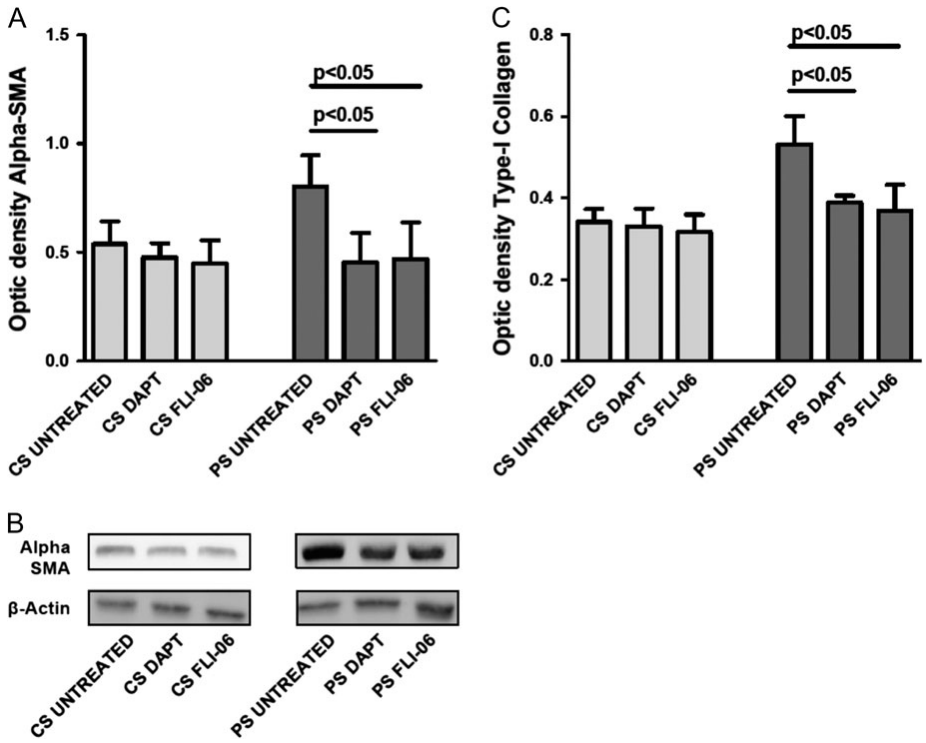

Figure 3 Evaluation of fibrosis markers before and after treatment with γ-secretase inhibitors. Evaluation of fibrosis markers α-SMA and type-I collagen in *Cs* and *Ps* before and after treatment with γ-secretase inhibitors (DAPT and FLI-06) assessed by western blot of cell lysates for α-SMA (**B**) and by picro-sirius red staining for type-I collagen. The mean OD ratio for α-SMA/β-actin was calculated in each group of cells (**A**). The mean OD at 540 nm was used for type-I collagen testing and calculated in each group of cells (**C**). Statistical analyses were performed using a non-parametric test for matched samples (Wilcoxon matched pairs test). Error bars represent SEM. Arbitrary units are reported. α-SMA, α-smooth muscle actin.

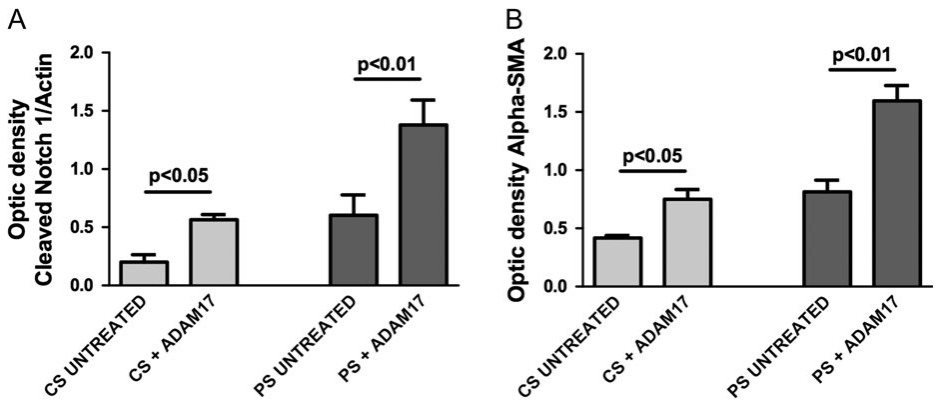

Figure 4 Evaluation of Notch-1 intracellular domain and α-SMA levels before and after stimulation with ADAM17 purified protein. NICD (**A**) and α-SMA levels (**B**) were analysed in *Cs* ($n = 8$) and *Ps* ($n = 8$) before and after stimulation with ADAM17 purified protein. Comparisons were performed using statistics for matched samples as appropriate. The mean OD ratio for Notch/β-actin and α-SMA/β-actin was calculated for both cell types. Error bars represent SEM. Arbitrary units are reported.

Indeed, the activation of Notch receptor is followed by two subsequent cleavages of the activated receptor, releasing the NICD, the active form of Notch proteins. In our study, the highest amounts of NICD were found in *Ps* and *Es* from DIE patients compared to *Cs* cells from disease-free patients. Recent research has shown that Notch signalling is crucial in endometrial decidualization and receptivity, and that both the loss and gain of Notch function may result in implantation impairment (Su *et al.*, 2016). The recent study by Su *et al.* (2016) also attributed

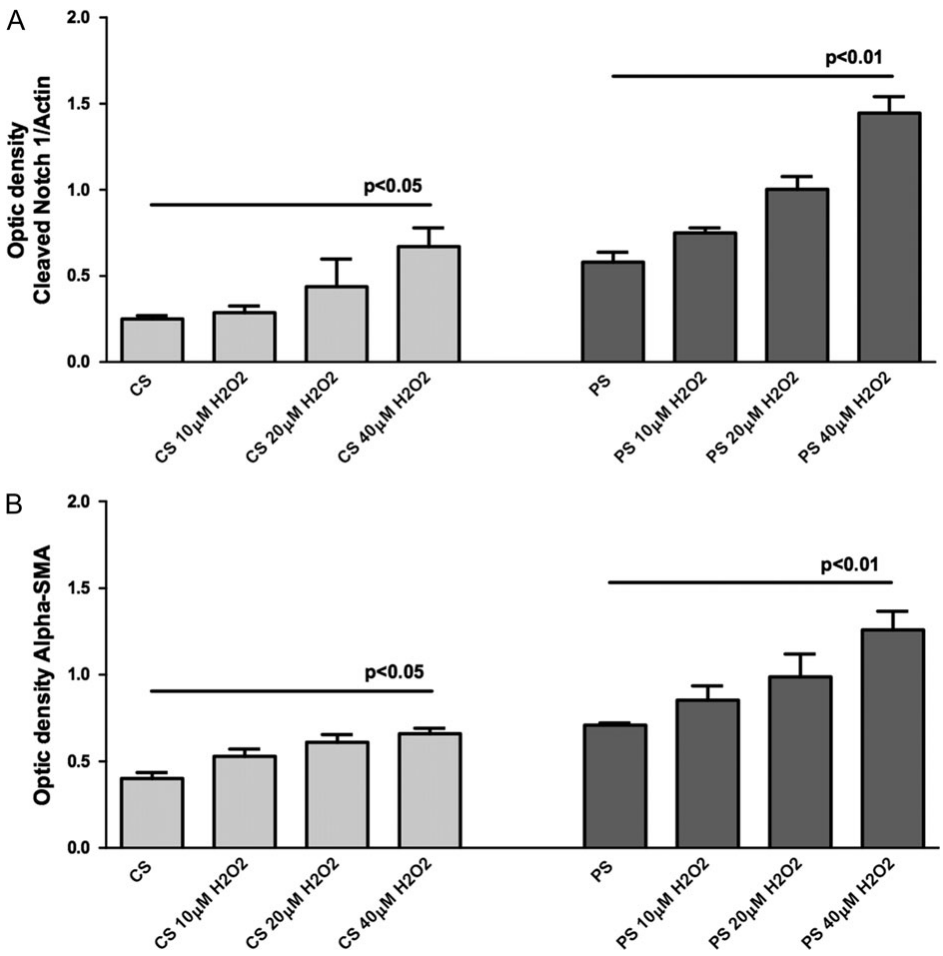

Figure 5 Evaluation of Notch-1 intracellular domain and α-SMA levels before and after incubation with increasing concentrations of hydrogen peroxide. Evaluation of Notch-1 intracellular domain (**A**) and α-SMA levels (**B**) in *Cs* ($n = 8$) and *Ps* ($n = 8$) in basal status and after incubation with increasing concentrations of hydrogen peroxide (H_2O_2). Statistical analyses were performed using Friedman test. Dunn's Multiple Comparison test was used for post hoc analysis. The mean OD ratio for Notch/β-actin and α-SMA/β-actin was calculated for both cell types. Error bars represent SEM. Arbitrary units are reported.

Notch with an interesting role in the regulation of progesterone receptor methylation, leading to a progesterone resistance profile. Our findings revealing Notch overexpression in *Es* and *Ps* cells from DIE patients compared to controls may present a relationship with progesterone resistance and the worse reproductive outcomes observed in patients with endometriosis. However, this was not the main target of our research and further studies are needed to draw valid conclusions regarding this specific point.

The NICD translocates into the nucleus and is directly involved in the transcriptional regulation of nuclear target genes that lead to fibrosis (Kavian *et al.*, 2010). The role of Notch signalling in fibrotic processes has been observed in several diseases and organs such as the skin (Kavian *et al.*, 2012a,b), the lung (Xu *et al.*, 2012), the kidney (Xiao *et al.*, 2014) or the liver (Chen *et al.*, 2012). Fibrosis is also a key feature of endometriosis, as this process is significantly associated with the most severe forms of endometriosis, especially DIE, and is observed in ectopic lesions (Clement, 2007). Indeed, the fibrosis markers α-SMA (a marker of myofibroblast differentiation) (Darby *et al.*, 1990) and type-I collagen were significantly increased in *Ps* cells from DIE patients compared with *Cs* cells from controls. To evaluate the magnitude of the role of Notch signalling in the fibrotic process that occurs in *Ps* cells from DIE patients, *Ps* and *Cs* cells were treated with DAPT and FLI-06, inhibitors of the γ-secretase complex that prevent Notch cleavage (Imbimbo, 2008). After treatment with these compounds, *Ps* cells presented a significant reduction of α-SMA and type-I collagen, while *Cs* cells with normal amounts of NICD did not show a decrease in either of the two fibrosis markers. These results reveal a hyperactivation of the Notch pathway in ectopic cells from DIE lesions, but not in endometrial cells from controls. For this reason, we believe that the Notch signalling pathway plays an important role in the fibrotic processes of DIE ectopic lesions as already observed in other pro-fibrotic disorders (Gonzalez and Medici, 2014). This observation not only provides a better understanding of the molecular mechanisms that take place in DIE lesions, but also opens new perspectives for therapeutic interventions, such as with γ-secretase inhibitors, that are currently under study in clinical trials (Andersson and Lendahl, 2014).

In order to reinforce the relationship between ADAM17, Notch and fibrosis in endometriosis we stimulated *Ps* and *Cs* cells with increasing concentrations of H_2O_2, ADAM17 purified protein and culture with their counterpart supernatant and vice-versa. These additional experiments showed that each intervention was able to increase Notch and

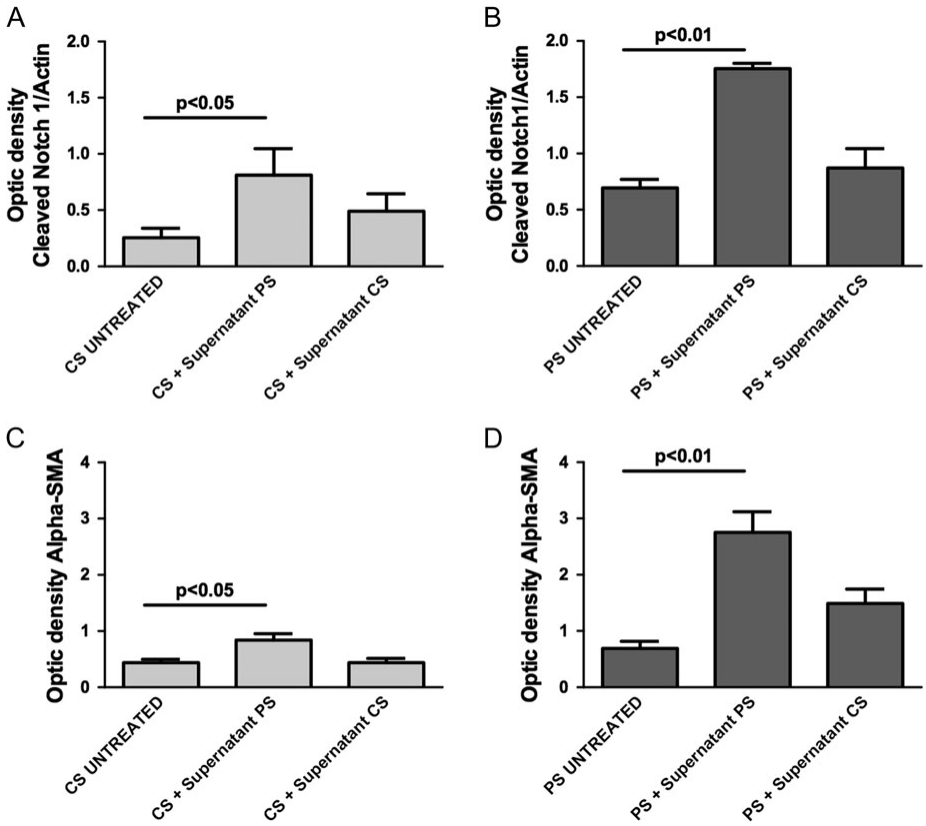

Figure 6 Evaluation of Notch-1 intracellular domain and α-SMA levels before and after incubation with supernatants. Comparison of Notch-1 intracellular domain (**A** and **B**) and α-SMA levels (**C** and **D**) in *Cs* ($n = 8$) and *Ps* ($n = 8$) before and after incubation with their own supernatant and their counterpart supernatant. Statistical analyses were performed using the Friedman test. Dunn's Multiple Comparison test was used for post hoc tests. The mean OD ratio for Notch/β-actin and α-SMA/β-actin was calculated for both cell types. Error bars represent SEM. Arbitrary units are reported.

α-SMA regardless of the origin of the cells, although this increase was more evident in *Ps* cells from DIE patients than controls (*Cs*).

The main strength of this study is based on the novelty of the topic and its accurate methodological design. As far as we are aware, this is the first study in endometriosis patients that links oxidative stress with the ADAM17/Notch signalling pathway and consequent fibrosis. Moreover, and according to the heterogeneity of the disease, patients were selected with well-defined clinical phenotypes. We only included patients with complete surgical evaluation of the peritoneal cavity, and thus, the endometriosis state was perfectly assessed, and patients were allocated to the endometriotic or the control group according to a previously validated classification (Chapron *et al.*, 2006). On the other hand, control patients were evaluated in the same way as endometriotic patients, and therefore, clinical and histopathological findings were also recorded and taken into account. In addition, the diversity of the pathologies present in our control patients reflects the most common benign conditions in gynaecology.

Nonetheless, this study may have some limitations and biases. First, the elevated proportion of patients with severe endometriosis in the study group is not a real representation of the prevalence of this condition among the general population. This selection bias occurs because the recruitment was performed in a highly specialized centre dealing with severe endometriosis. Nevertheless, we believe this consideration does not alter the main outcomes of the study. Second, the small sample size of endometrial biopsies used for each group of patients ($n = 8$) is a limitation of the study, and results should be interpreted with caution. Third, we also recognize that there is no ideal control group for studying peritoneal stress oxidative parameters in women with endometriosis. Our control group involved women operated for benign gynaecological conditions and some of these conditions (uterine fibroids, tubal infertility or ovarian cysts), might also be associated with altered peritoneal protein oxidative stress markers (Santulli *et al.*, 2013; Markowska *et al.*, 2015). Speaking against this possibility is the fact that we found clear statistically significant differences between women with and without endometriosis. Fourth, despite the fact that 40% of the patients were under preoperative hormonal treatment, data on the menstrual phases of the rest of the study participants were not available. However, it has been previously demonstrated that AOPP levels do not differ between menstrual phases (Santulli *et al.*, 2015). Additionally, we analysed the effect of hormonal treatment in AOPP and ADAM17 expression, and we failed to find any differences in AOPP and ADAM17 levels between patients who used hormonal treatment and those who did not.

In conclusion, this is the first study that demonstrates a relationship between increased oxidative stress and hyperactivation of the ADAM17/Notch signalling in women with endometriosis. Our findings

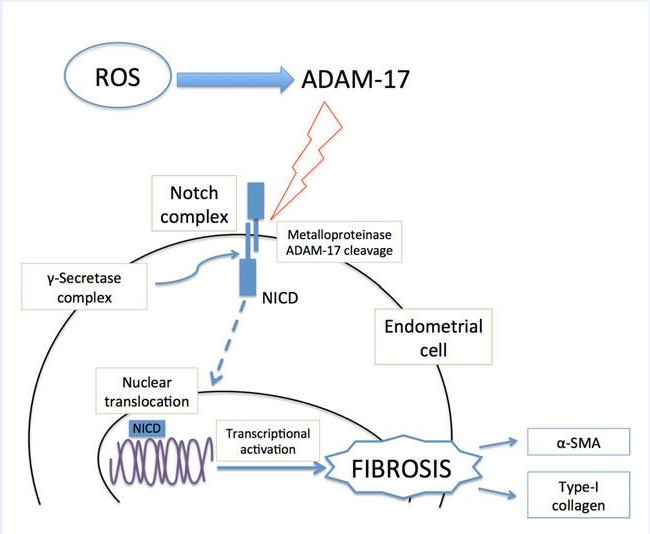

Figure 7 Representation of the relationship between oxidative stress, ADAM17 activity, Notch cleavage and fibrosis in the pathogenesis of endometriosis. Increased ROS led to a higher activity of ADAM17 metalloproteinase. Consequently, ADAM17 produces a proteolytic release of the active form of Notch proteins (also called Notch intracellular domain (NICD)) and its transport to the nucleus, where it induces transcriptional activation of genes related to fibrosis. ROS, reactive oxygen species.

could partly explain the major fibrosis events that occur in the disease, as well as the immune dysfunction related to the natural killer ligand shedding phenomenon. Although this study provides new evidence on the pathogenesis of endometriosis, further studies are needed to better understand the genesis and to find therapeutic targets to help in the treatment of this complex disorder.

Supplementary data

Supplementary data are available at *Molecular Human Reproduction* online.

Authors' roles

I.G.-F., P.S., C.C. and F.B. contributed to the conception and design of the study. I.G.-F., P.S. and S.C. collected the data, performed the experiments and developed the statistical analyses. I.G.-F., P.S. and F.B. wrote the manuscript. F.C., C.C. and F.B. revised the manuscript for important intellectual content. All authors approved the final manuscript.

Funding

Grants from University Paris Descartes, INSERM and Fundación Alfonso Martín Escudero.

Conflict of interest

The authors declare no conflict of interest.

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Article 2

Soluble ligands for the NKG2D receptor are released during endometriosis and correlate with disease severity

PLOS | ONE

RESEARCH ARTICLE

Soluble Ligands for the NKG2D Receptor Are Released during Endometriosis and Correlate with Disease Severity

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Citation: González-Foruria I, Santulli P, Chouzenoux S, Carmona F, Batteux F, Chapron C (2015) Soluble Ligands for the NKG2D Receptor Are Released during Endometriosis and Correlate with Disease Severity. PLoS ONE 10(3): e0119961. doi:10.1371/journal.pone.0119961

Academic Editor: Andrew Horne, University of Edinburgh, UNITED KINGDOM

Received: October 15, 2014

Accepted: January 18, 2015

Published: March 16, 2015

Data Availability Statement: All relevant data are within the paper and its Supporting Information files.

Funding: This work was supported by grants from University Paris Descartes, INSERM, and Fundación Alfonso Martín Escudero. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing Interests: The authors have declared that no competing interests exist.

Abstract

Background

Endometriosis is a benign gynaecological disease. Abundant bulk of evidence suggests that patients with endometriosis have an immunity dysfunction that enables ectopic endometrial cells to implant and proliferate. Previous studies show that natural killer cells have a pivotal role in the immune control of endometriosis.

Methods and Findings

This is a prospective laboratory study conducted in a tertiary-care university hospital between January 2011 and April 2013. We investigated non-pregnant, younger than 42-year-old patients (*n*= 202) during surgery for benign gynaecological conditions. After complete surgical exploration of the abdominopelvic cavity, 121 women with histologically proven endometriosis and 81 endometriosis-free controls women were enrolled. Patients with endometriosis were classified according to a surgical classification in three different types of endometriosis: superficial peritoneal endometriosis (SUP), ovarian endometrioma (OMA) and deep infiltrating endometriosis (DIE). Peritoneal fluid samples were obtained from all study participants during the surgery in order to detect soluble NKG2D ligands (MICA, MICB and ULBP-2). When samples with undetectable peritoneal fluid levels of MICA, MICB and ULBP-2 were excluded, MICA ratio levels were significantly higher in endometriosis patients than in controls (median, 1.1 pg/mg; range, 0.1–143.5 versus median, 0.6 pg/mg; range, 0.1–3.5; p=0.003). In a similar manner peritoneal fluid MICB levels were also increased in endometriosis-affected patients compared with disease-free women (median, 4.6 pg/mg; range, 1.2–4702 versus median, 3.4 pg/mg; range, 0.7–20.1; p=0.001). According to the surgical classification, peritoneal fluid soluble MICA, MICB and ULBP-2 ratio

levels were significantly increased in DIE as compared to controls (p=0.015, p=0.003 and p=0.045 respectively). MICA ratio levels also correlated with dysmenorrhea (r=0.232; p=0.029), total rAFS score (r=0.221; p=0.031) and adhesions rAFS score (r=0.221; p=0.031).

Conclusions

We demonstrate a significant increase of peritoneal fluid NKG2D ligands in women with endometriosis especially in those cases presenting DIE. This study suggests that NKG2D ligands shedding is a novel pathway in endometriosis complex pathogenesis that impairs NK cell function.

Introduction

Endometriosis is a benign chronic gynaecological disease characterized by the presence of endometrium-like tissue-glands and stroma- outside the uterine cavity [1]. This condition may affect up to 10–15% of women in childbearing-age, causing pelvic pain [2], and infertility [3].

Although endometriosis was first reported by Carl von Rokitansky more than a hundred years ago [4], the pathogenesis of this condition is still not clear [5]. Sampson's theory of retrograde menstruation is probably the most accepted among scientific community, though this explanation cannot adequately account for all the pathogenesis of the disease [6]. Previous studies report that retrograde menstruation occurs in >90% of women [7], nevertheless, the incidence of endometriosis is much lower in general population, which means that there should be physiological mechanisms with scavenging capacity that are able to eliminate the ectopic endometrial implants from the menstrual reflux.

Abundant bulk of evidence suggests that patients with endometriosis have an immunity dysfunction that enables ectopic endometrial cells to implant and proliferate [8–11]. Notwithstanding, the onset and progression of the disease is probably not only an immune issue, but the result of a complex series of processes that lead to the attachment of endometrial cells to the peritoneal surface [12], invasion and estrogen-related proliferation [13], vasculogenesis [14], angiogenesis and finally chronic inflammation [15,16]. Chronic inflammation is associated with an overproduction of prostaglandins [17], metalloproteinases, cytokines and chemokines, creating a self-supporting loop that mantains and amplificates the progression of the disease [18–20]. Once the process is started, many profibrotic mediators also play a role in the fibrogenesis associated with endometriosis [21].

From an immunological point of view, it has been shown that circulating natural killer (NK) cells are capable of destroying endometrial cells [22]. Thus, it has been proposed that NK cells may have a pivotal role in the immune control of endometriosis [23,24]. Additionally, many studies demonstrate that NK cells in blood and peritoneal fluid of endometriosis patients present a decreased cytotoxicity to autologous and heterologous endometrium [25,26].

The activity of NK cells is regulated through the signals from their receptors in a highly complex manner. NKG2D is an activating C-type lectin-like NK cell receptor involved in the elimination of infected or transformed cells. Upon binding to the corresponding ligands, NKG2D triggers a cytotoxic response that activates NK cells.

There have been described multiple ligands for this receptor (NKG2DLs), which are members of the *major histocompatibility complex* (MHC) class I family. In humans these ligands are MHC class-I chain related proteins (MIC) A, MICB and UL16 binding protein (ULBP) 1–6

PLOS ONE | DOI:10.1371/journal.pone.0119961 March 16, 2015

[27–29]. Paradoxically, the presence of the soluble form of NKG2DLs generates an inhibitory action on NK cells. In fact, it has been shown that increased levels of soluble NKG2DLs are present in the sera of cancer patients and this mechanism has been proposed as a strategy for tumours to avoid immune surveillance [30,31].

Research in the mechanisms that lead to the immune dysfunction present in endometriosis is a key step to better understand the pathogenesis of the disease.

The aim of the present study is to find a novel pathway that impairs NK cell function and facilitates the implantation and growth of refluxed endometrial cells over the peritoneal surface. For this reason, we assayed peritoneal fluid NKG2DLs (MICA, MICB and ULBP-2) obtained from patients with histologically proven endometriosis and endometriosis-free women. These results were compared and correlated among them and with respect of the endometriosis phenotype and the severity of the disease.

Materials and Methods

Patients

The local ethics committee (CCPPRB: Comité Consultatif de Protection des Personnes dans la Recherche Biomédicale) of Paris Cochin approved the study protocol and all study participants gave informed written consent for the enrolment in the study protocol.

From January 2011 to April 2013, a continuous series of 202 women, younger than 42-years-old, who underwent a laparoscopy for gynaecological reasons in our centre had been recruited in this study. Clinical and biological data were prospectively collected. Excluded from this population were pregnant women, women with cancer and those who did not give consent to the study. Women were classified into two groups depending on laparoscopic findings [32,33]: the endometriosis group consisted of subjects with histologically proven endometriosis, while patients in the control group did not show any macroscopic sign of the disease after a meticulous exploration of the abdominal cavity during the surgical procedure.

Endometriosis was staged and scored (total, implant and adhesion scores) according to the revised American Fertility Society (rAFS) Classification [34]. Besides, patients with endometriosis were also staged according to their endometriosis phenotype. Based on histological findings, endometriotic lesions were classified into three phenotypes: peritoneal superficial endometriosis (SUP), ovarian endometrioma (OMA) and deep infiltrating endometriosis (DIE). As these three types of lesions are frequently associated and may coexist [35] patients with endometriosis were classified according to the most severe finding. Endometriotic lesions are usually ranked from the least severe to the most severe in SUP, OMA and DIE [36]. By definition, DIE patients were graded from the least to the most severe DIE lesion as follows: uterosacral ligament (s), vagina, bladder, intestine and ureter [37]. The patient's most severe localization was considered for grading.

The study analysis used a prospective managed database. For each patient, personal history data were obtained during face-to-face interviews, which were conducted by the surgeon the month before surgery. A highly structured previously published questionnaire was used for all patients [38,39]. The following items were recorded: age, parity, gravidity, height, weight, BMI, past history of hormonal and/or surgical treatment for endometriosis, existence of gynaecological pain symptoms (dysmenorrhea, deep dyspareunia, non-cyclic chronic pelvic pain—NCCPP-), gastrointestinal [40] and lower urinary tract symptoms. According to a previous publication, NCCPP is defined as intermittent or permanent pelvic pain not related to the menstrual cycle [41]. In order to evaluate the pain intensity preoperatively a previously validated 10-cm visual analog scale was used [42].

Collection of peritoneal fluid samples

Peritoneal fluid was taken during surgery from all the patients of the study (202). The samples were centrifuged at 800 g for 10 min at 4°C and supernatants were collected. Aliquots of the samples were stored at -80°C until needed for the analysis.

Measurement of NKG2D ligands concentration

MICA, MICB and ULBP-2 were assayed in the peritoneal fluid by an enzyme-linked immunosorbent assay (ELISA) (R&D Systems, Inc., Minneapolis, MN, USA), according to the manufacturer's recommendations. The range of determination for MICA and ULBP-2 was 62'5–4.000 pg/ml and for MICB 156–10.000 pg/ml. The results below the lower threshold levels were considered as 0 pg/ml for the statistical analysis. Each sample was tested in duplicate and reflected the mean of the two measurements

Peritoneal fluid protein concentration was measured in all the samples using the spectroscopic Bradford protein assay method [43].

In order to avoid biases owing to peritoneal fluid concentration or dilution at the moment of obtaining the sample, a ratio between the NKG2D ligands result (pg/ml) and the protein concentration (mg/ml) was calculated for each peritoneal sample individually (values are expressed in pg of NKG2D ligand/ mg of protein).

Statistical analysis

All data were collected in a computerized database and subsequently analyzed by Statistical Package for the Social Sciences Software (SPSS Inc., Chicago, IL, USA). When endometriosis and control samples were analyzed, Student's t-test was used for quantitative variables and Pearson's X2 or Fisher's exact test were performed for qualitative variables. Considering the non-Gaussian distribution of MICA, MICB and ULBP-2 levels, statistical analysis between the two groups was performed with the Mann-Whitney U test.

When more than two groups were compared, Kruskal-Wallis test was used. When group medians were significantly different by the Kruskal-Wallis test ($p<0,05$), pairwise comparisons were performed using the with Dunn's Multiple Comparison Test.

In the view of the number of samples with undetectable levels of MICA, MICB and ULBP-2, two different statistical analyses were performed, one including and the other excluding the samples with undetectable levels of these NKG2D ligands. Correlations between MICA, MICB and ULBP-2 levels in peritoneal fluid and surgical findings and clinical characteristics of disease severity, measured with semiquantitative variables, were examined using the non-parametric Spearman's rank correlation test. $P<0,05$ was considered statistically significant.

Results

Study Population

One hundred and twenty one endometriosis-affected women and 81 disease free women were recruited for this study. Their clinical and surgical characteristics are displayed in Table 1.

Following the endometriosis surgical classification [38,39] and based on the location of the worst lesion presented, the 121 histologically proven endometriotic patients were classified as follows: 41 (33.9%) SUP, 32 (26.4%) OMA (right 11; left 14; bilateral 7) and 48 (39.7%) DIE. Patients' distribution according to the worst lesion of DIE founded was the following: 13 (27.1%) uterosacral ligament(s), 7 (14.6%) vagina, 5 (10.4%) bladder, 21 (43.7%) intestine and 2 (4.2%) ureter. These 48 DIE patients presented a total of 113 histologically proven DIE lesions distributed as follows: 46 uterosacral ligament lesions, 15 vaginal lesions, 7 bladder

PLOS ONE | DOI:10.1371/journal.pone.0119961 March 16, 2015

Table 1. Baseline characteristics of participants.

Patient characteristics	Endometriosis (N = 121)	Controls (N = 81)	P
Age (years) [a]	30.8 ± 5.1	31.7± 5.36	0.267[t]
Height (cm) [a]	167.5 ± 6.1	164.1 ± 6.0	<0.001[t]
Weight (kg) [a]	59.5 ± 8.1	61.5 ± 9.9	0.162[t]
BMI (kg/m^2) [a]	21.2 ± 2.5	22.8 ± 3.4	0.001[t]
Parity [a]	0.2 ± 0.5	0.4 ± 0.7	0.093[t]
Gravidity [a]	0.4 ± 0.7	0.6 ± 1.0	0.123[t]
Preoperative hormonal treatment (n, %)	48 (40.0%)	33 (40.1%)	0.887[k]
Infertility (n, %)	39 (32.2%)	30 (37.0%)	0.479[k]
Duration (month) [a]	39.0 ± 30.1	30.0 ± 17.6	
Previous treatment for endometriosis:			
* hormonal treatment (n, %)*	68 (56.2%)	NA	
* previous surgery (n, %):	19 (15.7%)	NA	
* previous endometrioma's surgery (n, %)	11 (9.1%)	NA	
Preoperative painful symptoms scores: [a, b, c]			
Dysmenorrhea	6.3 ± 2.9	4.6 ± 2.9	<0.001[t]
Deep dyspareunia [$]	4.1 ± 3.3	2.4 ± 3.0	0.001[t]
Non-cyclic chronic pelvic pain	2.9 ± 3.2	1.7 ± 2.6	0.011[t]
Gastrointestinal symptoms	3.2 ± 3.3	0.9 ± 1.9	<0.001[t]
Lower urinary symptoms	1.4 ± 2.8	0.2 ± 0.9	<0.001[t]
rAFS Classification:			
Mean implants score rAFS [a, d]	11.3 ± 11.2	NA	
Mean adhesions score rAFS [a, d]	9.6 ± 16.3	NA	
Mean total score rAFS [a, d]	21.2 ± 23.1	NA	
rAFS stage (n, %): [d]		NA	
I	37 (30.6%)		
II	16 (13.2%)		
III	26 (21.5%)		
IV	42 (34.7%)		
Surgical classification:			
Superficial endometriosis (n, %)	41 (33.9%)	NA	
Endometrioma (n,%)	32 (26.4%)	NA	
Endometrioma size (cm): [a]		NA	
Right	4.9 ± 2.8		
Left	4.8 ± 3.1		
Endometrioma laterality (n, %):		NA	
Bilateral	7/32 (21.9%)		
Right	11/32 (34.4%)		
Left	14/32 (43.7%)		
DIE lesions (n, %) [e]	48 (39.7%)	NA	
Mean number of DIE lesions [a]	2.3 ± 1.5	NA	
Total number of DIE lesions (n, %):		NA	
1	13/48 (27.1%)		
2	11/48 (22.9%)		
≥3	24/48 (50.0%)		

(*Continued*)

Table 1. (*Continued*)

Patient characteristics	Endometriosis (N = 121)	Controls (N = 81)	P
Anatomical distribution of DIE (n, %): [e, b]		NA	
USL	35/48 (72.9%)		
Vagina	15/48 (31.2%)		
Bladder	7/48 (14.6%)		
Intestine	23/48 (47.9%)		
Ureter	2/48 (4.2%)		
Worst DIE lesion (n, %): [e]		NA	
USL	13/48 (27.1%)		
Vagina	7/48 (14.6%)		
Bladder	5/48 (10.4%)		
Intestine	21/48 (43.7%)		
Ureter	2/48 (4.2%)		

[a] Data are presented as mean ± SD;
[b] Sometimes more than one for the same patient;
[c] Visual analogue scale (VAS);
[d] Score according to the American Fertility Society Classification (34)
[e] According to a previously published surgical classification for deeply infiltrating endometriosis (DIE) (37)
[t] Student's t-test;
[k] Pearson's chi-square test;
[$] 4% of patients have no sexual intercourse at the moment of the surgery.
* <5% missing data
NA: not applicable
USL: uterosacral ligaments.

doi:10.1371/journal.pone.0119961.t001

lesions, 43 intestinal lesions (1 intestinal lesion in 10 patients and more than 1 intestinal lesion in 13 patients) and 2 ureteral lesions. The mean (± SD) number of DIE lesions per patient was 2.3 ± 1.5 (range 1–6).

The indications for surgery in the 81 endometriosis-free women recruited in the study were the following: uterine fibroids (29 patients, 35.8%), non-endometriotic benign ovarian cysts (21 patients, 25.9%), tubal infertility (15 patients, 18.6%), pelvic pain (7 patients, 8.6%) and other indications (9 patients, 11.1%).

There were no differences in age, gravidity, parity and infertility between the endometriosis group and control group. Body mass index (BMI) was significantly lower in endometriotic patients than controls (P = 0.001). The percentage of patients with preoperative hormonal treatment was similar between the two groups (Table 1).

MICA, MICB and ULBP-2 levels

MICA, MICB and ULBP-2 levels were measured in peritoneal fluid of all the patients included in the study (n = 202). MICA, MICB and ULBP-2 were detected in the peritoneal fluid of 140 (69.3%), 196 (97.0%) and 34 (16.8%) study participants respectively.

MICA was detected in 82 (67.8%) endometriosis-affected women and in 58 (71.6%) controls (P = 0.641). MICB resulted positive in 118 (97.5%) patients with endometriosis and in 78 (96.3%) endometriosis-free patients (P = 0.685). Detection rate for ULPB-2 was significantly higher in endometriotic women as compared to controls (27 (22.3%) vs. 7 (8.6%), respectively; P = 0.012) (S1 Table).

Table 2. Statistical analyses for peritoneal NKG2D ligands (MICA, MICB and ULBP-2) ratio levels in women with endometriosis and controls.

xTable 2: NKG2D Ligands ratio levels in women with endometriosis and controls.			
	Endometriosis	**Controls**	**p**
Peritoneal MICA (with)	(n = 121) 0.48 (0.0–143.5)	(n = 81) 0.23 (0.0–3.5)	0.060 [u]
Peritoneal MICA (without)	(n = 82) 1.06 (0.1–143.5)	(n = 58) 0.56 (0.1–3.5)	0.003 [u]
Peritoneal MICB (with)	(n = 121) 4.87 (0.0–4702)	(n = 81) 3.40 (0.0–20.1)	<0.001 [u]
Peritoneal MICB (without)	(n = 118) 4.61 (1.2–4702)	(n = 78) 3.48 (0.7–20.1)	0.001 [u]
Peritoneal ULBP-2 (with)	(n = 121) 0.00 (0.0–5.2)	(n = 81) 0.00 (0.0–4.2)	0.014 [u]
Peritoneal ULBP-2 (without)	(n = 27) 0.36 (0.1–5.2)	(n = 7) 0.52 (0.1–4.2)	0.551 [u]

Analyses were performed both including samples with undetectable levels of NLG2D ligands (i.e. below lower limit of detection of the assay) and without these samples.

Results are expressed as median (range)

Values are expressed in pg/mg

u. Statistical analysis was performed with the Mann-Whitney U Test.

doi:10.1371/journal.pone.0119961.t002

According to the surgical classification, among endometriosis-affected women, MICA was detected in 25 (60.9%) SUP, 24 (75.0%) OMA and 33 (68.7%) DIE patients (P = 0.566). MICB became positive in 39 (95.1%) SUP, 31 (96.8%) OMA and 48 (100%) DIE patients (P = 0.543), while ULBP-2 was detected in 7 (17.1%) SUP, 10 (31.2%) OMA, 10 (20.8%) DIE patients (P = 0.026) (S1 Table).

When samples with undetectable peritoneal fluid levels of MICA, MICB and ULBP-2 were excluded, MICA and MICB levels were significantly higher in endometriosis patients than in controls (median, 1.1 pg/mg; range, 0.1–143.5 vs. median, 0.6 pg/mg; range, 0.1–3.5; P = 0.003 for MICA; median, 4.6 pg/mg; range, 1.2–4702 vs. median, 3.4 pg/mg; range, 0.7–20.1; P = 0.001 for MICB). In contrast, ULPB-2 levels in endometriosis patients were not significantly different from controls (median, 0.4 pg/mg; range, 0.1–5.2 vs. median, 0.5 pg/mg; range, 0.1–4.2; P = 0.551). In a similar way, when considering all the samples of endometriosis patients and controls, the levels of MICA (median, 0.5 pg/mg; range, 0.0–143.5 vs. median, 0.2 pg/mg; range, 0.0–3.5; P = 0.060), MICB (median, 4.9 pg/mg; range, 0.0–4702 vs. median, 3.4 pg/mg; range, 0.0–20.1; P< 0.001) and ULBP-2 (median, 0.0 pg/mg; range, 0.0–5.2 vs median, 0.0 pg/mg; range, 0.0–4.2; P = 0.014) resulted higher in the endometriosis group than in the control group, although for MICA the differences did not reach statistical significance. (Table 2 and Fig. 1).

According to the surgical classification, the medians of detectable MICA, MICB and ULBP-2 levels in DIE, OMA, SUP and control patients are depicted in Fig. 2. MICA (P = 0,015), MICB (P = 0.002) and ULBP-2 (P = 0.045) levels were significantly different between groups. A post-hoc test showed a significant increase in peritoneal MICA and MICB levels in DIE patients with the most severe forms of the disease compared to controls (median, 1.2 pg/mg; range, 0.1–143.5 vs. median, 0.6; range, 0.1–3.5; P< 0.05; median, 5.0 pg/mg; range, 1.4–4702 vs median, 3.6 pg/mg; range, 0.7–20.1; P< 0.01 for MICA and MICB respectively). In addition MICB levels were also significantly increased in SUP patients versus controls (median, 5.6 pg/mg; range, 1.2–35.1 vs. median, 3.6 pg/mg; range, 0.7–20.1; P <0.05). When post-hoc test was performed for ULBP-2 there were significant differences between DIE and SUP patients (median, 0.9 pg/mg; range, 0.2–5.2 vs. median, 0.2 pg/mg; range, 0.1–0.5; P< 0.05).

When all the samples were included in the analysis according to the surgical classification, MICA levels did not show differences between the groups (P = 0.100). However, MICB (P = 0.001) and ULBP-2 (P = 0.026) peritoneal levels were significantly different among groups. The post-hoc test showed for MICB significantly increased values in DIE and SUP

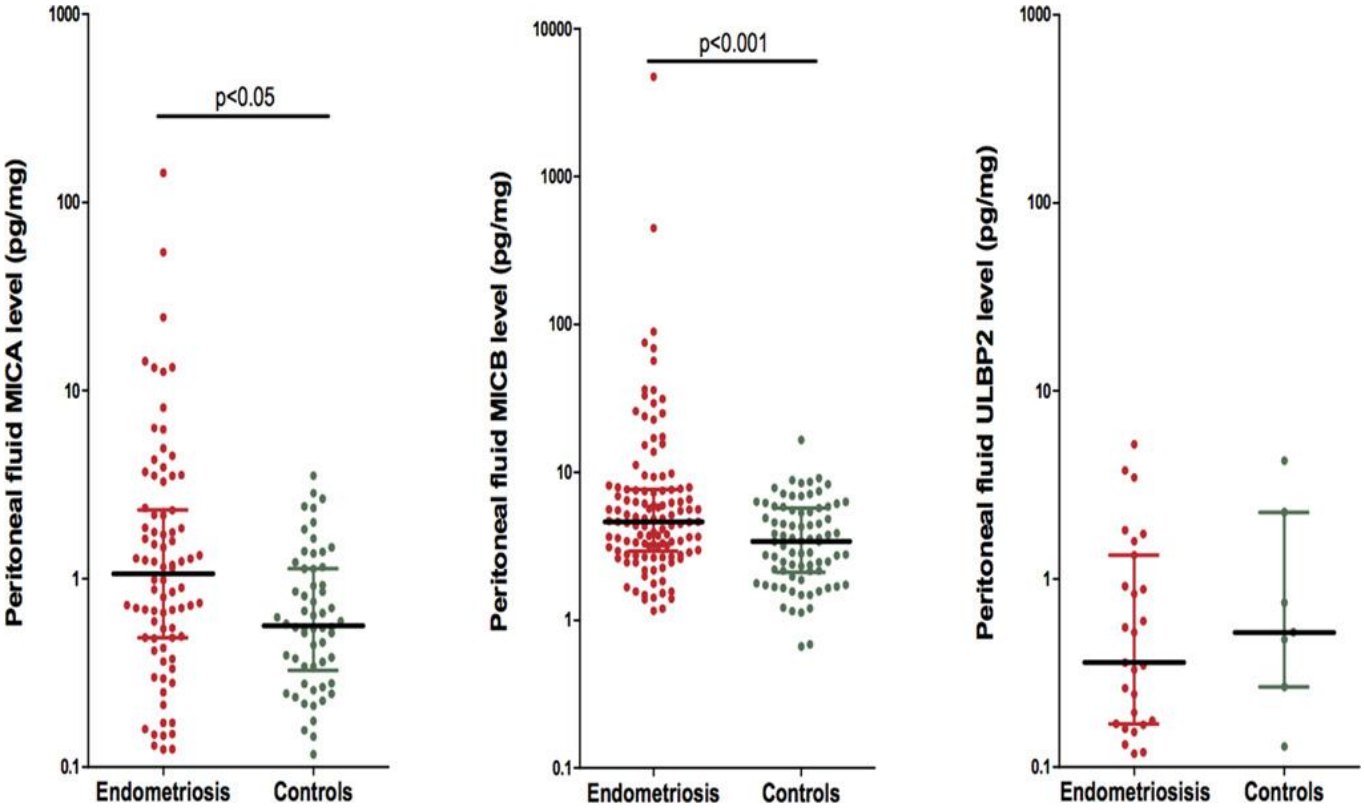

Fig 1. Peritoneal NKG2D ligands levels measured by ELISA in patients with endometriosis and in controls after exploration of the abdominopelvic cavity. Samples with undetectable levels of NKG2D ligands have been excluded. The peritoneal fluid MICA and MICB ratio levels among groups was significantly different by the Mann Whitney test (P = 0.035 and P<0.001 respectively). Peritoneal NKG2D ligands ratio values are represented on a logarithmic scale as a scatter dot plot. The medians with their interquartile range are reported.

doi:10.1371/journal.pone.0119961.g001

patients compared to controls (median, 5.0 pg/mg; range, 1.4–4702 vs. median, 3.4 pg/mg; range, 0.0–20.1; P< 0.01; median, 5.4 pg/mg; range, 0.0–35.1 vs. median, 3.4 pg/mg; range, 0.0–20.1; P< 0.05 for DIE and SUP patients respectively). The same analysis for ULBP-2, showed higher values in the OMA group in comparison to controls (median, 0.0 pg/mg; range, 0.0–1.8 vs. median, 0.0 pg/mg; range, 0.0–4.2; P <0.05) (S2 Table).

Clinical correlations with peritoneal fluid MICA, MICB and ULBP-2 levels

Clinical, surgical and biological correlations with peritoneal fluid MICA, MICB and ULBP-2 levels in women with endometriosis are expressed in S3 Table.

MICA correlated with MICB (r = 0.466; P <0.001) and ULBP-2 (r = 0.540; P = 0.009). In addition, MICA presented a clinical correlation with dysmenorrhea (r = 0.232; P = 0.029). There was also a positive correlation between MICA and Total rAFS score (r = 0.221; P = 0.031) and with Adhesions rAFS score (r = 0.221; P = 0.031). ULBP-2 levels also correlated with Adhesions rAFS score (r = 0.217; P = 0.034).

MICA correlated not only biologically with other NKG2DLs, but also clinically with pain score and surgically with some surgical findings corresponding to the severity of the disease.

MICA correlations with MICB, ULBP-2, Total rAFS score and Dysmenorrhea are depicted in S1 Fig.

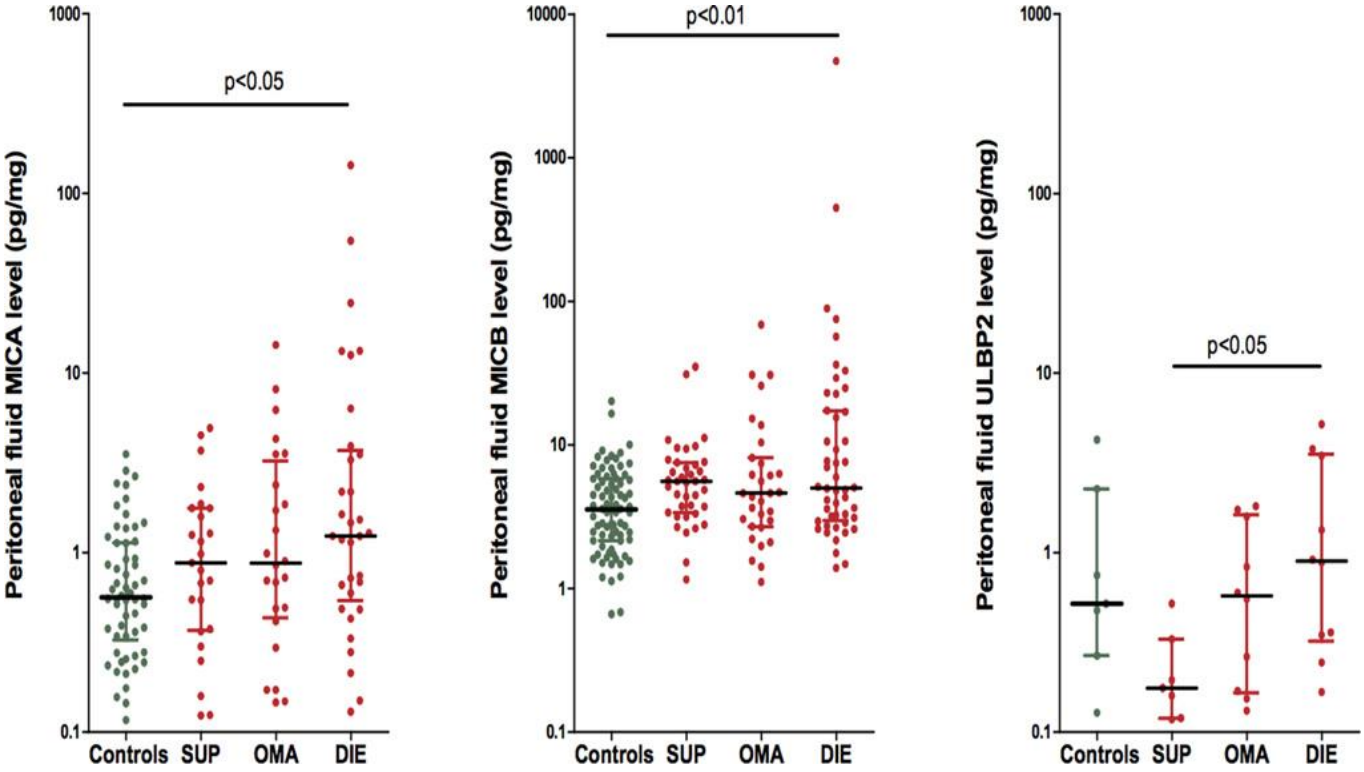

Fig 2. Peritoneal NKG2D ligands levels measured by ELISA in patients with endometriosis and in controls according to the surgical classification of endometriosis. Samples with undetectable levels of NKG2D ligands have been excluded. The peritoneal fluid MICA and MICB ratio levels among groups [DIE (n = 48), OMA (n = 31); SUP (n = 39) and controls (n = 78)] was significantly different by the Kruskal-Wallis test (P = 0.015 and P = 0.002 respectively). Post hoc test were performed using the with Dunn's Multiple Comparison Test. Peritoneal NKG2D ligands ratio values are represented on a logarithmic scale as a scatter dot plot. The medians with their interquartile ranges are reported.

doi:10.1371/journal.pone.0119961.g002

Discussion

This is the first report on NKG2DLs levels in peritoneal fluid of women with endometriosis. Our study shows that MICB levels are significantly elevated in peritoneal fluid of patients with endometriosis as compared to endometriosis-free women, especially in those patients with the most severe forms of the disease. Concerning peritoneal fluid MICA levels, our results demonstrate higher levels in the endometriosis group than controls, though when considering all the samples (detectable and undetectable values), the differences did not reach statistical significance. The same results could not be obtained for ULBP-2. We found a strong biological correlation between MICA and other NKG2DLs (MICB and ULBP-2). In addition, significant correlations were also found between MICA and clinical characteristics of the disease. High peritoneal fluid levels of MICA were positively correlated with dysmenorrhea, total rAFS score and adhesions rAFS score [34], though these clinical associations had a moderate correlation coefficient.

The strength of this study is based on the originality of the topic and its methodological design: (i) To the best of our knowledge, this is the first study on the assessment of peritoneal fluid NKG2DLs levels in endometriotic patients. Moreover, this study evaluates a large number of peritoneal fluids from patients who were submitted to a gynaecological intervention (121 endometriotic women and 81 controls); (ii) Taking into consideration the heterogeneity of the disease [36], patients were selected with very well-defined clinical phenotypes. The inclusion of patients in the study was performed following a thorough abdominopelvic cavity exploration.

All surgical and histological findings were recorded in order to grade the severity of the disease and to distribute each patient according to the former described classification [37]. As shown in previous publications, the wide anatomical heterogenity of endometriosis demands a concrete surgical workup in order to best classify the patients studied [44,45]; (iii) Free-disease patients were clinically and surgically evaluated in the same manner as endometriotic patients. Besides, the wide variety of pathologies present in our control patients exhibits the most common benign conditions in gynaecological patients.

Despite an accurate study design, our results may be subject to certain biases and limitations: (i) though detection rates for MICA and MICB in peritoneal fluids using the ELISA Kit were high (69 and 97% respectively), the rate of detection for ULBP-2 resulted far below from the others (17%). This high rates of undetectable levels, hampers the statistical analysis of data and its interpretation. The rate of undetectable ULBP-2 levels is a result in itself, though the way these undetectable results should be managed for statistical analysis is not clear so far. For this reason, and in order to avoid possible weak points related to the presence of undetectable levels of ULBP-2, MICA and MICB, we carried out two separated statistical assessments: one including and the other excluding the undetectable values [20]. Nevertheless, despite having a low detection rate for ULBP-2, when a detectable value of this ligand was obtained, the likelihood of belonging to an endometriotic patient was significantly higher. These findings lead us to believe an existing role of ULBP-2 in the pathogenesis of the disease; (ii) The high proportion of women with severe endometriosis in our study, does not reflect the real prevalence of disease severity among general population. This selection bias occurs because patients were recruited in our centre, which is a referral centre specialized in the care of grave endometriosis. However, this consideration should not alter the main results of the study; (iii) Despite the control population used in this study display the most common benign gynaecological disorders, it is not clear whether these conditions may modify peritoneal fluid NKG2DLs levels in those patients. The ideal patient used as control should be that without any illness at all. However, nowadays it is hardly feasible to obtain peritoneal fluid from healthy patients. For this reason, and although our results are clear regarding the differences in peritoneal fluid NKG2DLs between endometriotic patients and women without the disease, we believe these data must be interpreted with regard; (iv) The MICA ELISA assay may not equally detect all the different soluble MICA molecules; in fact, more than 60 allelic variants have been described. Our finding that soluble MICA could be detected in endometriotic and control patients suggests that this system was applicable for our cohort of endometriotic patients. As specific allelic variants of MICA exist, we cannot rule out that the differences observed between groups do not reflect various concentrations of different soluble MICA variants. For this reason, special caution should be paid for the use of this ELISA system for widely polymorphic MICA; (v) The ULBP2 ELISA used in this study exhibits some cross reactivity with an other member of the UL16 binding protein family (ULBP), ULBP6. Some ULBP members like ULBP1, 2, 3 and 6 are glycosylphosphatidylinositol-anchoring molecules without transmembrane domain that can be proteolytically shed from the surface of cells. One limitation of our study is that there are no strictly ULBP2-specific or ULBP6-specific ELISA available due to the high sequence identity between ULBP2 and ULBP6. Accordingly, studies of soluble ULBP2 likely measured both ULBP2 and ULBP6. Although we cannot exclude that soluble ULBP6 have been detected along with soluble ULBP2 in our patients, the probability for this to occur is expected to be low in our particular clinical situation.

It is nowadays accepted that the immune system actively contributes to the homeostasis of the peritoneal cavity. Women with endometriosis show some kind of immune dysfunction that plays a role in the pathogenesis of the disease [8–11]. Among many other immunological factors, it has been shown that NK lymphocytes display reduced cytotoxicity against endometrial

PLOS ONE | DOI:10.1371/journal.pone.0119961 March 16, 2015

cells [22,26,46]. Thus, it has been suggested that NK cells are implicated in the scavenging of the refluxed endometrial debris in the peritoneum. Some authors have shown that endometriosis surgical treatment does not improve NK cell function [47], which implies that NK dysfunction may be more a cause than a consequence of endometriosis itself. In addition, hormonal treatments such as GnRHa or dienogest, do improve NK cells activity [48–50].

NK cells function is regulated in a highly complex manner by the balance of activating and inhibiting receptors on they surface. It has been stated that an increase in the killer inhibitory receptor (KIR) on NK cells, leads to a reduction of their cytotoxic activity over the endometrial cells [51,52]. Other NK receptors such as natural cytotoxicity receptors have also been studied and showed differences between patients and controls [53].

NKG2D receptor is a major activating receptor on NK cells [54]. NKG2DLs interaction triggers an activating signal and promotes cytotoxic response of the cell expressing the ligand. NKG2DLs are not expressed in most normal healthy cells, but they are frequently overexpressed in infected or transformed cells, acting as a threating signal that enables the immune system to recognize stressed cells [55]. Cell surface expression of NKG2DLs is down-regulated by proteolytic shedding mediated by metalloproteases that come from oxidative stress [30,31,56]. The liberation of soluble forms of the ectodomain of NKG2DLs not only decreases the expression of the ligands on target cells but it also generates the internalization and lysosomal degradation of NKG2D receptor, leading to a paradoxical inhibition of NK cells function. In fact, NKG2DLs shedding has been shown to be a tumour escape mechanism. Elevated NKG2DLs levels have been detected in sera of cancer patients, and correlations among these levels and the stage of the disease or the tumour progression have been demonstrated [27,57,58].

In a similar way to tumours, endometriosis avoids immune surveillance in different manners. In our experience, we believe that the elevation of peritoneal fluid NKG2DLs levels in endometriotic patients is one of the mechanisms of immune dysfunction present in this disorder. In fact, the most severe forms of the disease present higher levels of peritoneal fluid NKG2DLs, leading to a major NK cell dysfunction.

The observed increase in soluble forms of NKG2DLs in peritoneal fluid means a lower expression of these ligands in ectopic endometrial cells surface, heading toward greater evasion from NK cells recognition. Additionally, the rise in soluble NKG2DLs levels further inhibits NK cells cytotoxicity, and consequently NK cell dysfunction becomes more pronounced. According to this rationale, peritoneal invasion and proliferation of ectopic endometrial cells turns out to be more facile when NKG2DLs shedding occurs. The clinical correlations observed in our study reinforce even more our hypothesis.

In conclusion, we demonstrate for first time an increase of peritoneal fluid NKG2DLs in endometriotic patients compared to controls. This study suggests that NKG2DLs shedding is a novel pathway in endometriosis complex pathogenesis that impairs NK cell function. Nevertheless, further studies are needed to determine the genesis of NKG2DLs increase, opening new possible therapeutic targets on this complex disorder [59].

Supporting Information

S1 Fig. Correlation analysis of peritoneal fluid MICA levels with MICB, ULBP and with total rAFS score and dysmenorrhea score. Non-parametric Spearman's correlation tests was used to assess correlations.
(TIFF)

S1 Table. NKG2D Ligands qualitative results according to the surgical classification. [k] Pearson's chi-square test; [f] Fisher exact test.
(DOCX)

S2 Table. Peritoneal NKG2D Ligands levels according to the surgical classification. Analyses were performed both including samples with undetectable levels of NKG2D ligands (i.e. below lower limit of detection of the assay) and without these samples. Results are expressed as median (range). Values are expressed in pg/mg. SUP: superficial peritoneal endometriosis. OMA: Endometrioma. DIE: Deeply infiltrating endometriosis. k. Statistical analysis was performed using Kruskal-Wallis test. Post hoc test were performed using the with Dunn's Multiple Comparison Test. *Significantly different from control women ($p < 0.05$). ** Significantly different from control women ($p < 0.01$). ***Significantly different from SUP ($p < 0.05$).
(DOCX)

S3 Table. Correlation analysis of peritoneal fluid MICA, MICB and ULBP-2 levels and clinical data in women with endometriosis. Note: Pain intensity was evaluated preoperatively using a previously validated 10-cm VAS scale. (26) rAFS: according to The Revised American Fertility Society classification of endometriosis (1). * Only in DIE patients.
(DOCX)

Author Contributions

Conceived and designed the experiments: FB CC. Performed the experiments: IGF SC. Analyzed the data: PS. Wrote the paper: IGF PS. Contributed to critically revising the intellectual content of the work: FC FB CC.

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