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ORIGINAL ARTICLE

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

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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.

[†]Equal contribution.

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.

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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

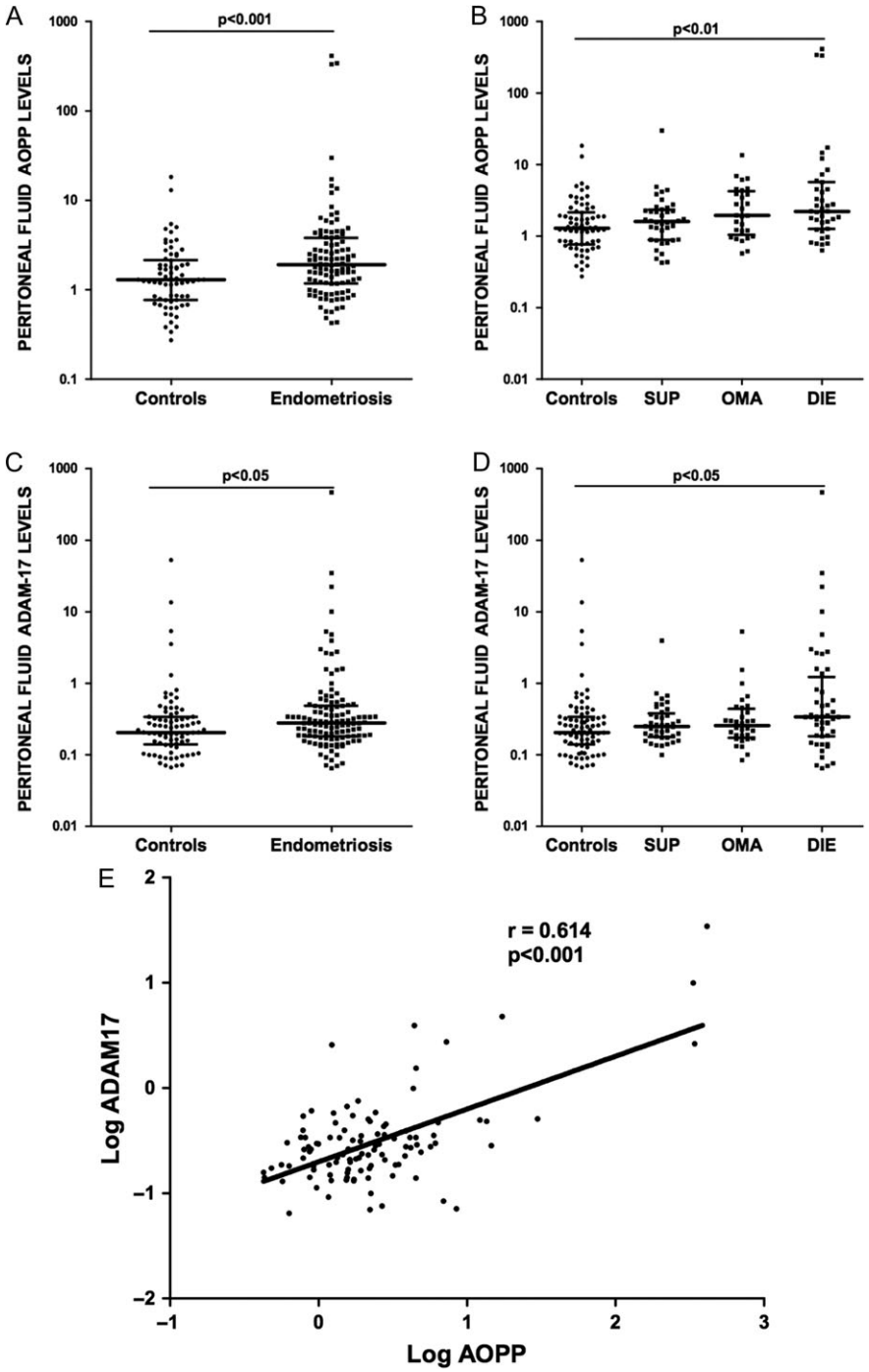

Figure 1 AOPPs and ADAM17 levels in patients with endometriosis and controls. (**A**) AOPP measured in PF by ELISA in patients with endometriosis ($n = 121$) and in controls ($n = 81$) after exploration of the abdominopelvic cavity. (**B**) AOPP levels measured by ELISA according to the surgical classification 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.

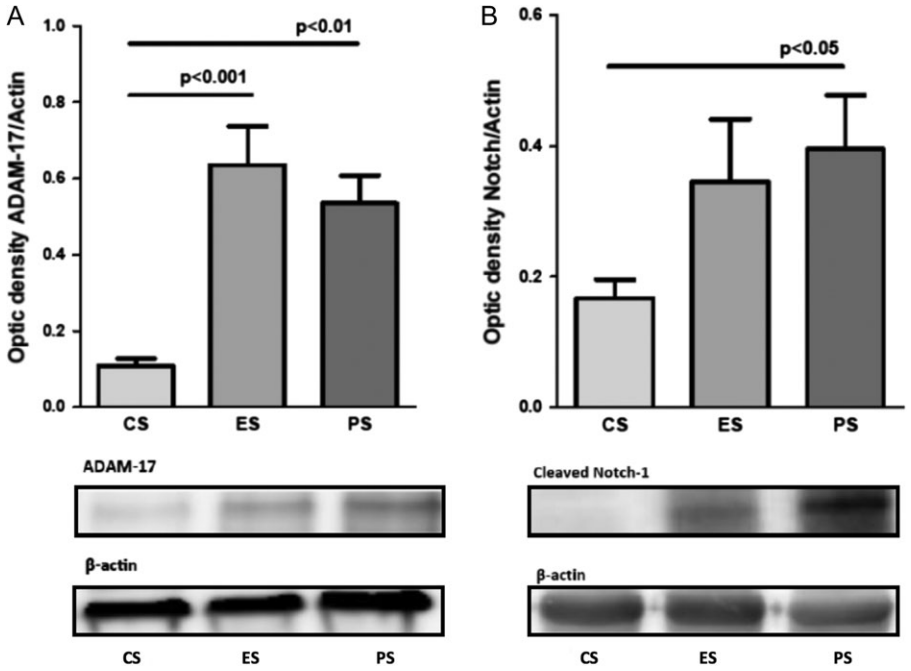

Figure 2 ADAM17 and Notch-1 intracellular domain (NICD) levels in stromal endometrial cells of control women and patients with DIE. Differences in ADAM17 activity (**A**) and NICD levels (**B**) among eutopic stromal endometrial cells (*Cs*) of control patients ($n = 8$), and stromal cells of eutopic (*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).

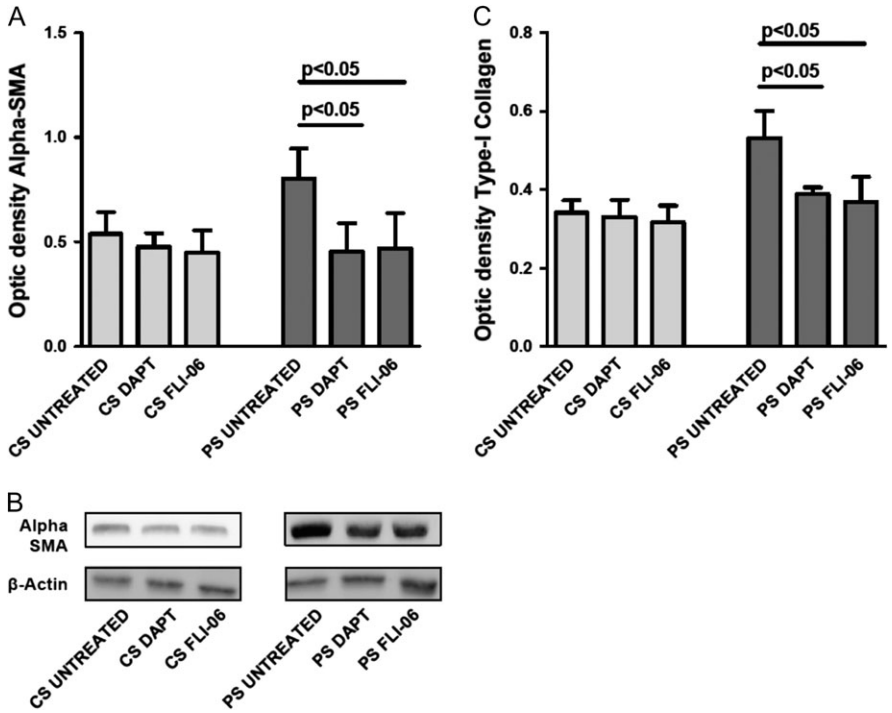

Figure 3 Evaluation of fibrosis markers before and after treatment with γ-secretase inhibitors. Evaluation of fibrosis markers α-SMA and type-I collagen in *Cs* and *Ps* before and after treatment with γ-secretase inhibitors (DAPT and FLI-06) assessed by western blot of cell lysates for α-SMA (**B**) 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.

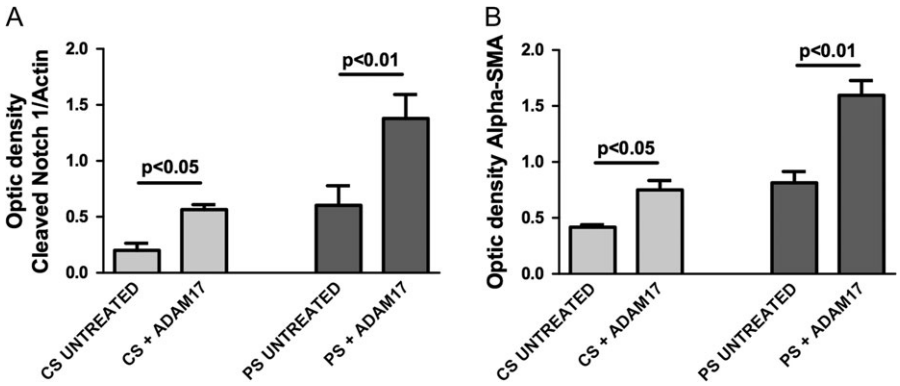

Figure 4 Evaluation of Notch-1 intracellular domain and α-SMA levels before and after stimulation with ADAM17 purified protein. NICD (**A**) and α-SMA levels (**B**) were analysed in *Cs* ($n = 8$) and *Ps* ($n = 8$) before and after stimulation with ADAM17 purified protein. Comparisons were performed 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

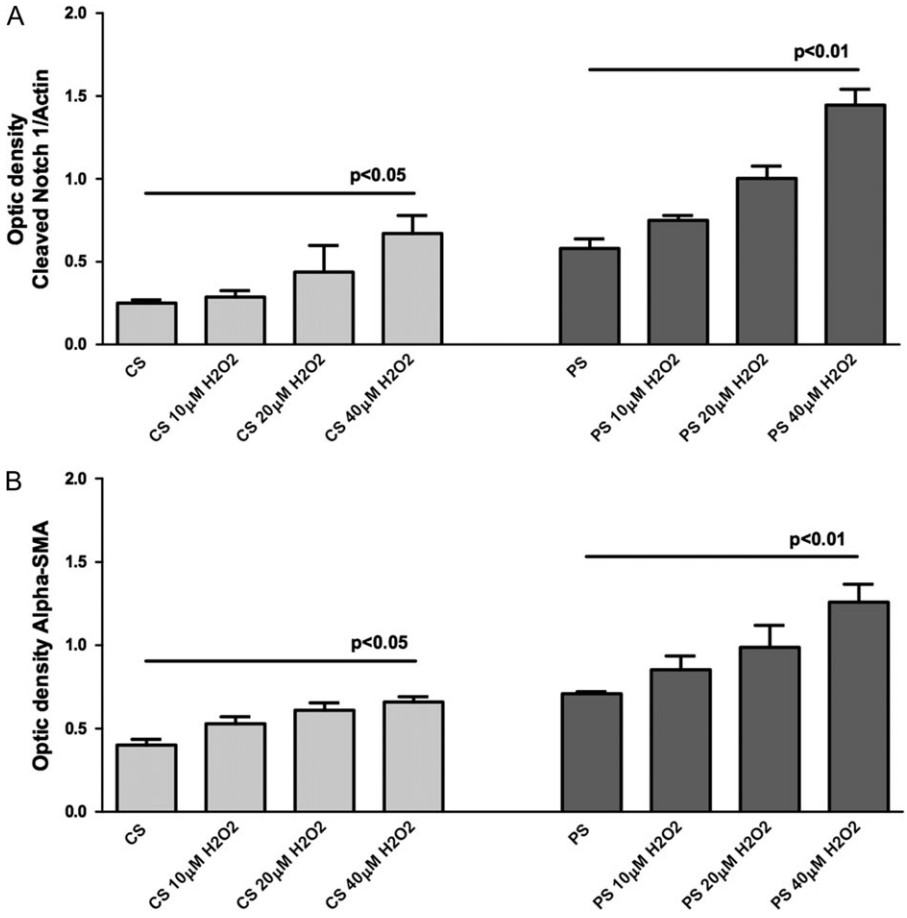

Figure 5 Evaluation of Notch-1 intracellular domain and α-SMA levels before and after incubation with increasing concentrations of hydrogen peroxide. Evaluation of Notch-1 intracellular domain (**A**) and α-SMA levels (**B**) in *Cs* ($n = 8$) and *Ps* ($n = 8$) in basal status and after incubation with 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

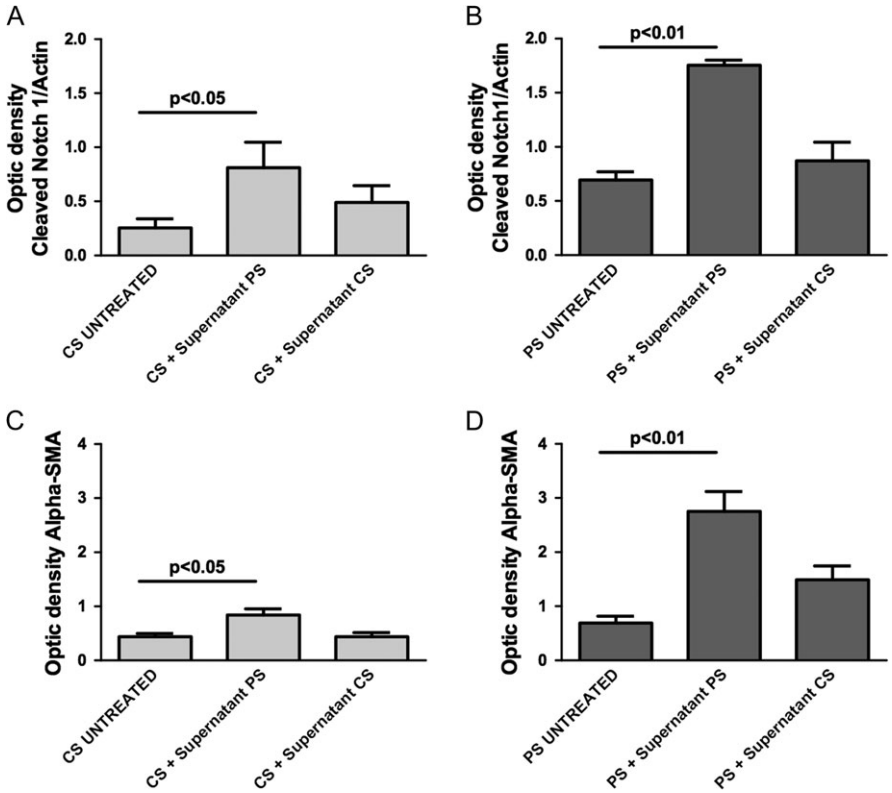

Figure 6 Evaluation of Notch-1 intracellular domain and α-SMA levels before and after incubation with supernatants. Comparison of Notch-1 intracellular domain (**A** and **B**) and α-SMA levels (**C** and **D**) in *Cs* ($n = 8$) and *Ps* ($n = 8$) before and after incubation with their own supernatant 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

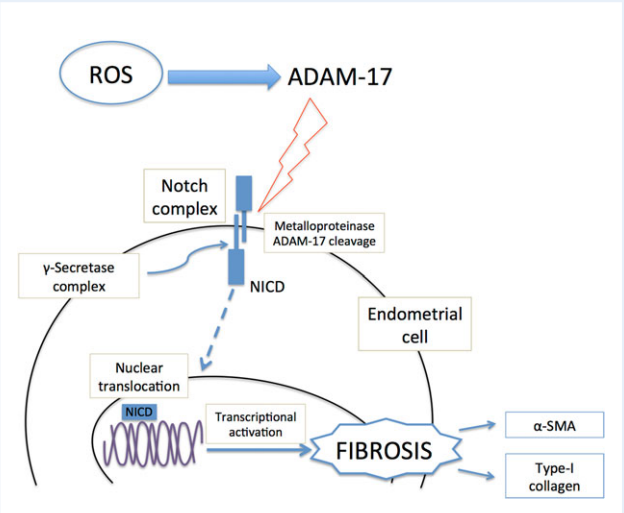

Figure 7 Representation of the relationship between oxidative stress, ADAM17 activity, Notch cleavage and fibrosis in the pathogenesis of endometriosis. Increased ROS led to a higher activity of ADAM17 metalloproteinase. Consequently, ADAM17 produces a proteolytic release of the active form of Notch 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.

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Conflict of interest

The authors declare no conflict of interest.

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