Menstrual endometrial supernatant may induce stromal endometriosis in baboons

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1. ABSTRACT

We tested the hypothesis that endometriosis can be induced in baboons more successfully by intra-pelvic injection of the pellet of menstrual endometrium when compared to its supernatant. Menstrual endometrium, separated into pellet (n = 5) and supernatant fractions (n = 8), or phosphate buffered saline (1 ml, n = 7, controls) was injected laparoscopically into the pelvis. During laparoscopy 25 days later, the number (p = 0.027) and surface area (p <0.0001) of endometriosis-like lesions were significantly higher in the pellet group, than in the supernatant group, or in the control group. Histological typical endometriosis was present only in the endometrial pellet group (1/15), whereas stromal endometriosis was observed in both the pellet group (6/15), and the supernatant group (6/20). Peritoneal endometrial like glands were observed in both the endometrial pellet group (3/15), and in the supernatant group (1/20). In conclusion, we confirmed our hypothesis that endometriosis can be induced in baboons more successfully by intrapelvic injection of the pellet of menstrual endometrium when compared to its supernatant.

2. INTRODUCTION

Endometriosis is a gynaecological disease characterised by the presence of endometrial - like glands and stroma outside the uterine cavity. The clinical presentation depends on the location (peritoneal, ovarian or rectovaginal) and the extent of the disease. Clinical symptoms may include but are not limited to cyclic pelvic pain, dysmenorrhea, dyspareunia and infertility (1). The natural process of disease development is still poorly explored. Although the aetiology is not precisely known, endometriosis is proposed to occur when refluxed endometrial cells end up into the peritoneal cavity during menstruation (2). It has long been known that the menstrual effluent contains viable sloughed endometrial cells that are able to survive and implant (3,4). Retrograde menstruation is believed to contribute significantly towards the development of endometriotic lesions (2). However, previous studies have reported a low prevalence and quantity of endometrial cells in peritoneal fluid (PF) during menstruation (5;6) and a high (80%) rate of adhesion between endometrial cells and autologous peritoneum *in vitro* (7), both in women with and without endometriosis.

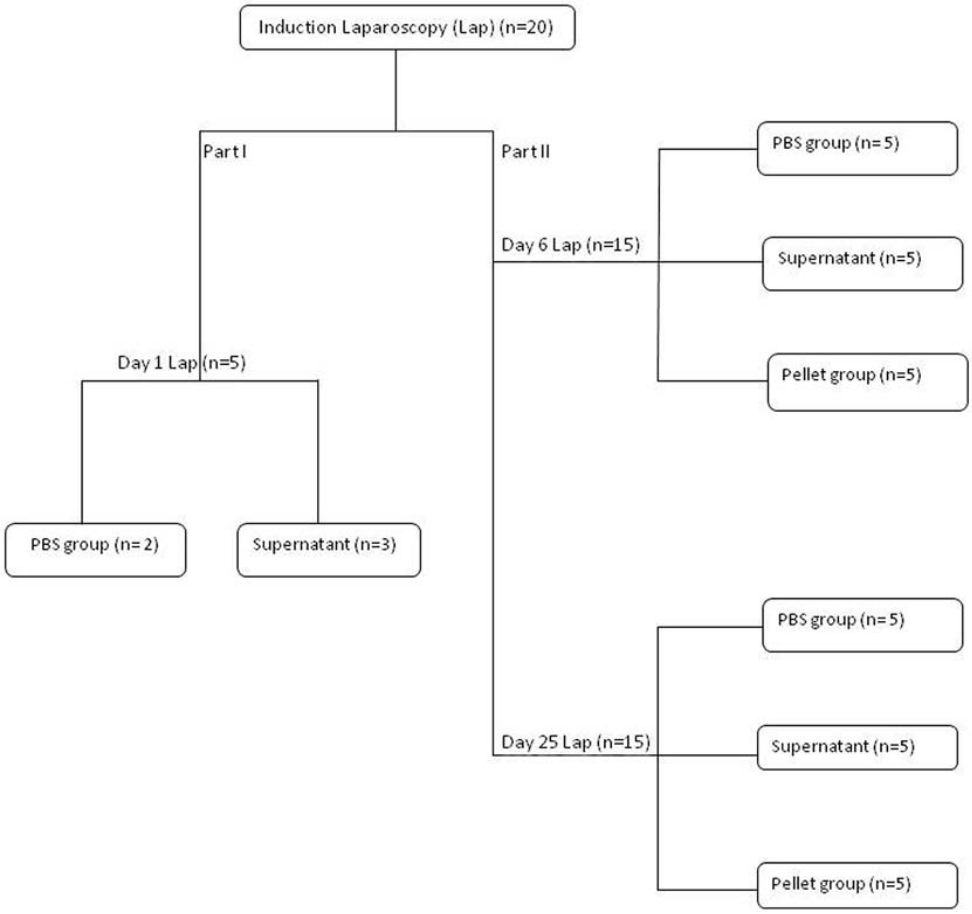

Figure 1. Flowchart on induction method and staging laparoscopies in 20 baboons. Part I (n=5) Day 0: Laparoscopic induction with menstrual EM supernatant (n=3) or with PBS (n=2) Day 1: Staging laparoscopy with biopsies from menstrual EM supernatant group (3 baboons, 14 biopsies) and from PBS group (2 baboons, 8 biopsies) Part II (n=15) Day 0: Laparoscopic induction with menstrual EM pellet (n=5), menstrual EM supernatant (n=5) or with PBS (n=5) Day 6: Staging laparoscopy without biopsies Day 25: Staging laparoscopy with biopsies from menstrual EM pellet group (5 baboons, 15 biopsies), menstrual EM supernatant group (5 baboons, 12 biopsies) and from PBS group (2 baboons, 3 biopsies)

These data suggest that the development of endometriosis may be influenced by other factors in menstrual flow other than endometrial cells in peritoneal fluid. Indeed, according to the induction theory, endometriosis may be caused by peritoneal metaplasia, possibly induced by substances that are present in peritoneal fluid at the time of menstruation (8;9;10). Metaplasia may play a pivotal role in disease development (11;12;13), and menstrual effluent can induce severe morphologic alterations in cultured mesothelial cells *in vitro* (14), but the induction theory has never been tested in a preclinical relevant animal model like nonhuman primates.

In baboons, endometriosis can be successfully induced surgically by intrapelvic injection of menstrual endometrium (15), allowing the investigation of the *in vivo* interaction between menstrual endometrium, peritoneum and peritoneal fluid in the pathogenesis of endometriosis. According to the Sampson hypothesis, it is assumed that this induction process is caused by the cellular fraction of injected endometrium (2) but it is also possible that the supernatant component plays a role in the induction process (8). The aim of this study was to test the hypothesis that intrapelvic injection of the pellet part of menstrual endometrium has the capacity to induce endometriosis more successfully than intrapelvic injection of the supernatant part of menstrual endometrium in baboons.

3. MATERIAL AND METHODS

A total of 20 adult female baboons (*Papio Anubis*), captured in the wild and kept in captivity at the Institute of Primate Research (IPR, Kenya) were used in the study. The animals were all kept in quarantine for 3 months during which period they were screened for tuberculosis , simian T-lymphotrophic Virus – 1 and simian immunodeficiency Virus. They were housed indoors with natural lighting in group cages and fed on commercial food pellets with fruits and vegetable supplementation three times a week and water *ad libitum*. All animal procedures and care had been conducted in accordance with the Institute of Primate Research standard operating

Table 1. Effects of cellular menstrual endometrial pellet, acellular menstrual endometrial (EM) supernatant and PBS (control) on the development and degree of induced endometriosis in baboons 25 days post induction

Groups	Number of lesions	Surface area lesion (mm^2)	rAFS	Stages of endometriosis
EM Pellet (Group 1)				
2852	21	331,75	8	II
2945	31	108	6	II
2955	25	192,25	8	II
2753	21	82,75	4	I
2804	28	567,25	20	III
EM Supernatant (Group 2)				
2952	6	23.25	4	I
2950	18	53.75	4	I
2948	6	48	4	I
2938	9	5.0	2	I
2879	4	1.75	1	I
PBS (Group 3)				
2883	0	0	0	0
2910	0	0	0	0
2630	0	0	0	0
2687	2	3	1	I
2693	5	3	1	I

procedures. The Institutional Scientific Evaluation and Review Committee and the Animal Care and Use Committee of Institute of Primate Research approved the study and and the Ethical Commission for Animal Research of the University of Leuven.

3.1. Induction of endometriosis

Anaesthesia and laparoscopies were carried out as described previously (16), and an overview of all laparoscopies and biopsies is shown in Figure 1. The induction laparoscopy was performed on the first or second day of menstruation. During this laparoscopy all animals were screened for the presence of pelvic abnormalities and found to have a normal pelvis. Transcervical curettage with a Novak curette was performed as previously described (15). Endometrium (1000±250mg) obtained by transcervical curettage during the first 2 days of menstruation was separated into pellet and supernatant by spinning at 2400rpm for 10 minutes. The baboons were divided into three groups according to the material used for intrapelvic seeding: endometrial pellet group (n=5), endometrial supernatant group (n=8) and control group, seeded with phosphate buffered saline (PBS, 1ml, n=7). These materials were seeded under laparoscopic vision using an 18-gauge needle, as reported previously (15). Induction sites were standardized to the uterovesical fold, urinary bladder, pelvic walls, anterior side of broad ligament, uterus and the pouch of douglas. The ovaries were excluded to prevent extensive development of tubal/ovarian adhesions, which could impair the inspection at follow-up laparoscopies.

3.1.1. Part 1: Videolaparoscopy after 1 day post induction

In the first part of the study, 5 baboons (3 from the endometrial supernatant group and 2 from the PBS group) received a videolaparascopy 1 day after induction to detect endometrial implants and, if present, to stage the extent of induced endometriosis according to type, localisation and size (length x width x depth) (Figure 1). This was done because it was not known whether the intrapelvic seeding of endometrial supernatant or PBS could result in endometriosis and it was felt to be important to document the pelvic status as soon as possible after induction. Baboons from the endometrial pellet group did not receive this laparoscopy since it was expected that they would certainly develop endometriosis, based on our previous work that the endometriosis induction rate after intrapelvic seeding of endometrium is 100% (15) and our current hypothesis that this induction success can be explained by peritoneal attachment of the cellular component of endometrium. A pelvic map was made with systematic photographic documentation of the pelvis during the laparoscopy. Representative biopsies (n = 14) of endometriotic lesions were collected from baboons in the endometrial supernatant group (n=8 from 3 baboons) and PBS group (n=6 from 2 baboons), and fixed in formalin for histological analysis. These baboons did not participate in part 2 of the study.

3.1.2. Part 2: Videolaparoscopy after 6 and 25 days post induction

In the second part of the study, videolaparoscopies were performed after 6 and 25 days post induction on 15 baboons that had not participated in Part 1 of the study (endometrial pellet group, (n=5); endometrial supernatant group, (n=5); PBS group, (n =5) to stage the extent of induced endometriosis after induction according to type, localisation and size (Figure 1). The internal genitalia and pelvic peritoneum were evaluated for the presence of endometriosis as previously described (16). A detailed pelvic map was made with systematic photographic and video documentation of the pelvis and endometriosis lesions were documented according to size (dimensions Length x width x depth), type and localisation. Endometriotic lesions were defined as white plaques with pigmented spots and red lesions included red-orange vesicles. Endometriotic lesions were characterised according to phenotype (red and white lesions), surface area and localisation. Red lesions were grouped as red vesicular, red hemorrhagic, red polypoid or red-white plaques. White lesions were divided into white plaques, white nodules or white clear vesicles. The extent of endometriosis was classified according to rAFS score system (17) after modification for baboon size (Table 1). Adhesions caused by individual endometriotic lesions were characterized either as dense or filmy, and the exact localisation of attachment and type of lesion causing the adhesion was documented. Endometriosis related adhesions

involving ovary, fallopian tube and cul-de-sac were noted separately and graded according to the revised classification system of the American Society for Reproductive Medicine (18). The surface area of an endometriotic lesion (and an endometriotic lesion-related adhesion) was determined by multiplying length (mm) x width (mm) and to calculate the revised American Fertility Society (rAFS) score and stage (Table 1), adapted for the baboon (15). No biopsies were taken at the laparoscopy 6 days post induction. During the laparoscopy performed 25 days after induction, 30 biopsies of endometriotic lesions were surgically obtained for histopathological confirmation of the disease from baboons in the endometrial pellet group (n=15 from 5 baboons), the endometrial supernatant group (n=12 from 5 baboons) and the PBS group (n=3 from 2 baboons), snap frozen in liquid nitrogen and stored at -80° C.

3.2. Histology

All 44 biopsies (Part 1: 14 paraffin-embedded biopsies; Part 2: 30 frozen biopsies) were serially sectioned (7μm thickness). Four serial sections were placed on silane-coated slides and every second subsequent slide was stained with hematoxylin and eosin for assessment. The rest of the slides were either kept frozen at -20 °C or were stored at room temperature in case of formalin fixed biopsies. This method of sectioning allowed every 28 μm of the biopsies to be stained. Based on the assumption that the smallest endometriotic lesion detectable at laparoscopy measures about 50μm in women (19), it is reasonable to state that, using this approach, all potentially detectable lesions could be identified using our approach. Typical endometriosis was defined as the presence of both endometrial glands and stroma in biopsies of suspected endometriotic lesions (20). Stromal endometriosis was defined as the presence of endometrial stromal cells only (20;21). Peritoneal endometrial-like glands were defined to be present if we observed epithelial structures without surrounding stroma containing a dilated glandular structure lined by columnar epithelium, suggestive for mullerian type epithelium (20;22).

In the first phase 100 serial sections of 7 μm thickness were prepared either from frozen biopsies (n = 30) using Cryostat HM 560 (Microm International, Merelbeke, Belgium) at -30^0 C or from each paraffin embedded biopsy (n = 14) using Leica Microtone (Type 2055 Autocut, Nussloch, Germany). The stained sections were examined under the light microscope (Leica DMLB, Wetzlar, Germany) for the presence of endometrial glands and stroma by 2 investigators (CMK and TMD) and results were confirmed independently by a senior pathologist (Prof Dr Philippe Moerman, MD, PhD). Additional serial sectioning was done for 33 biopsies (frozen (n = 22) and formalin fixed (n = 11)) that were histologically negative for typical or stromal endometriosis after initial sectioning, in the first phase, until they were histologically positive or until the biopsies were completely sectioned.

3.3. Immunohistochemistry

In 7 biopsies showing stromal endometriosis, obtained from baboons induced with either endometrial pellet (n = 2) or endometrial supernatant (n = 5), immunohistochemistry was performed to confirm the presence of endometrial stromal cells using CD10, an endometrial stromal marker (23;24). Representative paraffin embedded tissue (n = 4) sections were preheated for 2 hours at 55^0C, deparaffinized in toluene and rehydrated in ethanol. Tissue sections were retrieved in 0.01M Citrate buffer pH 6 for 1 hour at 80^0C. Frozen sections (n = 3) were used without antigen retrieval. Immunostaining was performed using a peroxidase NovoLink Polymer detection system (Novocastra, Newcastle Upon Tyne, UK) with minor modifications. Briefly endogenous peroxidase activity was neutralized using peroxidase block for 30 minutes followed by washing using 50mM Tris-buffered saline pH7.6 **(**TBS) for 5 minutes. Sections were incubated with protein block for 30 minutes and washed in TBS twice for 5 minutes. A mouse monoclonal antibody raised against human CD10 (1:100 dilution; Novocastra, Newcastle-upon-Tyne, UK) was used as primary antibody and incubated for 2 hours at room temperature. Subsequently, tissue sections were washed in TBS and blocked with post primary block for 30 minutes at room temperature followed by washing using TBS for 5 minutes. Incubated with Novolink polymer for 30 minutes and peroxidase activity was detected by 3, 3′-diaminobenzidine tetrahydrochloride (DAB) as the chromogen and Harris' haematoxylin used for counterstaining. Sections incubated without the primary antibody were included as negative control in all experiments while small intestine was used as the positive control. All slides were examined using a light microscope (Leica DMLB, Wetzlar, Germany).

3.4. Cytological analysis of endometrial supernatant and pellet

Since all endometrial tissue, including both supernatant and pellet, was used for induction in this study, no tissue was left over for cytological analysis. In order to test the hypothesis that endometrial supernatant does not contain endometrial cells, we analysed the cytological content of these tissues in an additional and independent study done in 4 baboons. Endometrium obtained by transcervical endometrial biopsy during day 1 (n=.2.) or day 2 (n=.2.) from 4 baboons (Papio Anubis, mean ± SD weight:13.6±1.03; median: 13.25; range:12.8 – 15.1), was centrifuged at 2400 rpm for 10 minutes to obtain a separate fraction of endometrial supernatant and endometrial pellet, using exactly the same protocol which had been used to treat the endometrium prior to intrapelvic injection in the main part of this study.

Separate smears of the endometrial pellet and endometrial supernatant fractions were made on microscopic glass slides. Firstly, the menstrual endometrial supernatant fraction was used to make smears after complete aspiration with special care ensuring that the tip of the pipette did not touch the menstrual endometrial pellet fraction at all. Secondly, the menstrual endometrial pellet fraction was aspirated and used for making smears. Smears were prepared from a small drop aspirated from either menstrual supernantant or menstrual pellet fraction and placed on a glass slide. Smears were made using another

Table 2. Analysis and comparison on the number of macroscopic endometriotic lesions and characterisation of lesion phenotype and surface area between menstrual endometrial pellet (Group 1), menstrual endometrial supernatant (Group 2) and PBS (Group 3)

	Group 1 (n=5)	**Group 2 (n=5)**	**Group 3 (n=5)**
No of lesions (median, range)	25,21-31; **total: 126**	6,4 -18; **total: 43**	1,0 - 5; **total: 8** ***P*=0.027**
No of lesion (red, white)	73,53	13,30	4,4
Surface area lesion (mm^2) (median, range)	2,0.25-250; **total: 757**	0.25,0.25-25;**total: 64.25**	1,0.25–2; **total**: **7** ***P*<0.0001**
Surface area lesion (mm^2) (red, white)	696.5, 60.5	37.25,27	4,3

slide laid on top of the drop and pulled apart quickly as the drop spreads from the weight of the slide. Cytological smears were fixed in 10% buffered formalin or acetone for 15 minutes for papanicolaou smear staining. The papanicolaou smear staining procedure was used on slides by first staining with haematoxylin, which stains the nuclei followed by counterstaining with OG-6 (orange g stain) and EA-50 (Multiple polychrome stain).

3.5. Statistical analysis

Results are expressed as median and range. The differences between groups, endometrial pellet group, endometrial supernatant group and PBS group was tested for statistical significance using one way analysis of variance (non-parametric) (Kruskal-Wallis) followed by Dunn's nonparametric t-tests and nonparametric Student *t* test (Mann-Whitney), Fisher Exact test where appropriate. A *P* value <0.05 was considered to be statistically significant. Statistical analysis was performed using the Prism 3 software (GraphPad, San Diego, CA, USA).

4. RESULTS

Baboon weight was similar before (mean ± SD: 13.8 ± 2.4, median: 13.20 and range; 9.95 – 17.6kg) and after induction of endometriosis (mean ± SD: 13.14 ± 2.3, median: 12.8, range; 9.5 - 17.3kgs).

4.1. Macroscopic endometriotic lesions: number, surface and ASRM classification

On day 1 after induction (Part 1 of the study), only a few macroscopic lesions compatible with endometriosis were observed in the 3 baboons from endometrial supernatant group (2, 4 and 2 lesions in each of the 3 baboons, respectively) and in the 2 baboons from PBS group (2 and 4 lesions in each of the 2 baboons, respectively).

On day 6 after induction (Part 2 of the study), dense adhesions were noted in all 5 baboons of the endometrial pellet group, but no adhesions were observed in baboons from the endometrial supernatant group (0/5) or PBS group (0/5). The total surface area of endometriosis lesions was significantly higher (p = 0.0024) in the endometrial pellet group (9.05±14.6, 3, 0.25-75) than in the endometrial supernatant group (3.1±5.5, 0.25, 0.25-25) or in the PBS group (0.25, 0.25, no range). The surface area of white lesions was significantly higher (p = 0.0061) in the endometrial pellet group (1.73±2.4 ,1, 0.25 - 9) than in the endometrial supernatant group (0.66±1.13, 0.25, 0.25 - 4) or in the PBS group (0.25, 0.25, no range).

On day 25 after induction (Part 2 of the study), dense adhesions were noted in all five baboons of the endometrial pellet group and no adhesions were observed in either endometrial supernatant group or PBS group. The number and surface area of endometriosis lesions and ASRM classification of endometriosis are shown in Table 1 and in Table 2. Examples of macroscopic white and red lesions are shown in Figure 2. The proportion of baboons with ASRM stage II or III endometriosis was significantly higher (p = 0.048) in the endometrial pellet group (4/5) than in the endometrial supernatant group (0/5). The total number of macroscopic lesions was significantly higher (Kruskal-Wallis test, p = 0.027) in the endometrial pellet group than in the endometrial supernatant group or in PBS group (Figure 3C). In a separate analysis (Mann-Whitney test) the total number of macroscopic endometriotic lesions was significantly higher in endometrial pellet group (p = 0.012) or endometrial supernatant group (p = 0.02) when compared to the PBS group (Figure 3C) separately. Furthermore, the total number of macroscopic endometriotic lesions was significantly higher in the endometrial pellet group (p = 0.01) than in the endometrial supernatant group (Figure 3C). The total surface area of macroscopic lesions was also significantly higher in the endometrial pellet group when compared to the endometrial supernatant group or PBS group ((p < 0.0001, Table 2). The number of endometriotic lesions and the endometriosis surface area for either red or white lesions were comparable on day 6 and day 25 after induction in all groups.

4.1.1. Red and white lesions

On day 25 after induction, both the number (p = 0.005) (Figure 3A) and the surface area (p = 0.005) of red lesions was significantly higher in the endometrial pellet group than in the endometrial supernatant group or in the PBS group. More (p = 0.0019) red lesions occupying a significantly larger surface area (p = 0.015) were observed in endometrial supernatant group when compared to PBS group (Table 2).

On day 25 after induction, both the number (p = 0.0015) (Figure 3B) and the surface area (p = 0.0023) of white lesions was higher in the endometrial pellet group than in the endometrial supernatant group or in the PBS group. The number of white lesions was significantly higher in the endometrial pellet group (p = 0.0112) when compared to the PBS group (Figure 3B). The surface area covered by white lesions was significantly higher (p = 0.007) in the endometrial pellet group than in the endometrial supernatant group (Table 2).

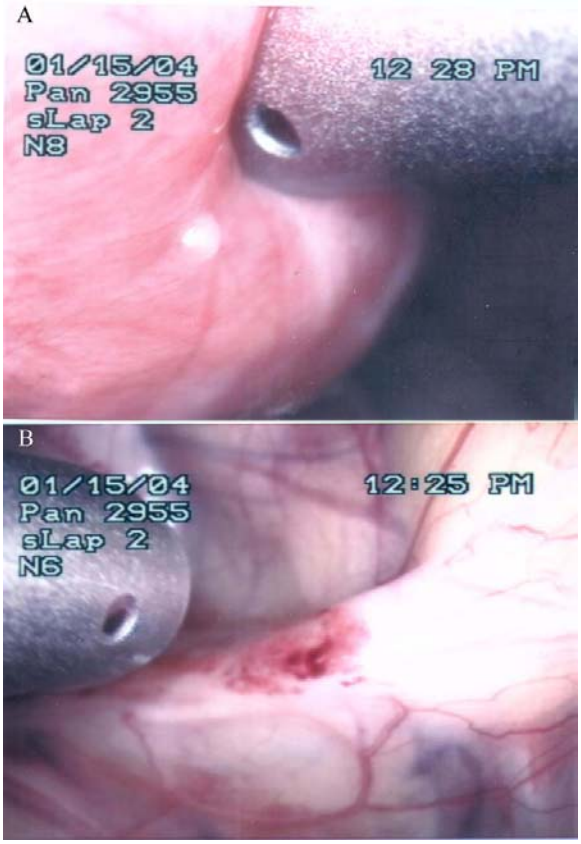

Figure 2. White (white peritoneal vesicle, Fig 2A) and, macroscopic red (hemorrhagic peritoneal area Fig 2B) lesions observed in baboon PAN 2955 6 days after induction with endometrial pellet.

4.2. Histological analysis

On day 1 after induction stromal endometriosis without glandular component was observed in 38% (3/8) biopsies from the endometrial supernatant group and in 0% (0/6) biopsies from the PBS group.

On day 25 after induction, the histological confirmation rate for typical endometriosis was only 6.7% (1/15) for the endometrial pellet group (Figure 4C) and was absent in the endometrial supernatant group 0% (0/12) and PBS group 0% (0/3). Stromal endometriosis without glandular component was observed in 40% (6/15) biopsies from the endometrial pellet group, in 25% (3/12) biopsies from the endometrial supernatant group (Figure 4B), and in 0% (0/3) biopsies from the PBS group. Peritoneal endometrial-like glands were observed in 20% (3/15) biopsies from the endometrial pellet group (Fig 4, D-F), in 8% (1/12) biopsies from the endometrial supernatant group (Fig 4A) and 0% (0/3) biopsies from the PBS group. Taking into account all biopsies (n=27) taken from baboons induced with endometrial pellet or supernatant on day 25, and including peritoneal endometrial-like glands without surrounding stroma as "histological endometriosis", the overall total histological confirmation rate was 52% (14/27), being 66% (10/15) in biopsies obtained after endometrial pellet induction and 33% (4/12) in biopsies obtained after endometrial supernatant induction. The biopsies obtained in the PBS group did not show any endometrial glands or stroma, but showed some degree of fibrosis .

4.3. Immunohistochemistry

The majority (71%, 5/7) biopsies with stromal endometriosis were stained positively for CD10, with cytoplasmic-specific staining (Figure 5D & F) being present in most stromal cells (Figure 5B).

4.4. Cytological analysis of menstrual endometrial pellet and endometrial supernatant

Cytological analysis of the menstrual endometrial pellet showed many clusters of endometrial cells, but cells could not be counted reliably individually as they were too packed together to allow individual counting (n=4). Endometrial supernatant samples contained some hemorrhage and loose endometrial-like stromal cells that appeared in a much lower density than in the pellet samples, with an estimated median of 1012 (range 190-3572) cells per high power field (at 200x magnification).

5. DISCUSSION

In this study, we confirmed our hypothesis that endometriosis can be induced in baboons more successfully by intrapelvic injection of the pellet of menstrual endometrium when compared to intrapelvic injection of its supernatant. After intrapelvic injection of menstrual supernatant, the induction of endometriosis was caused by a few endometrial cells, or by soluble substances present in the endometrial supernatant, as has been proposed in the induction theory for endometriosis.

According to the induction theory, tissues derived from coelomic epithelium, such as peritoneum, may differentiate into mullerian-directed epithelium, mediated by unknown soluble factors contained in retrograde menstrual flow (25). In earlier studies (8), it has been reported that dying endometrial tissue releases substances that activate undifferentiated mesenchyme to form endometrium in the peritoneum. In a rabbit model (9) a noncellular diffusible substance from adjacent endometrial implant was reported to have the capability to stimulate or induce epithelial differentiation of connective tissue into endometrial-like epithelium and glands, but histological interpretation of the data presented in this study (9) does not allow a clear-cut conclusion with respect to the endometrial nature of these lesions. In an *in vitro* model using human ovarian surface epithelial cells it has been demonstrated that endometriotic lesions can arise by a process of metaplasia from the ovarian surface epithelium, manifesting as a gradual and serial change from the adjacent mesothelial cells (13). In a previous study the surface mesothelium of pelvic peritoneum adjacent to active endometriosis was shown to undergo a sequence of reactive change from flat cell to cuboidal or columnar epithelium (26;27). *In vitro* studies also have shown that cells isolated from menstrual effluent induce changes on mesothelial morphology without causing apoptosis and necrosis (14), as well as in cancer cells (28).

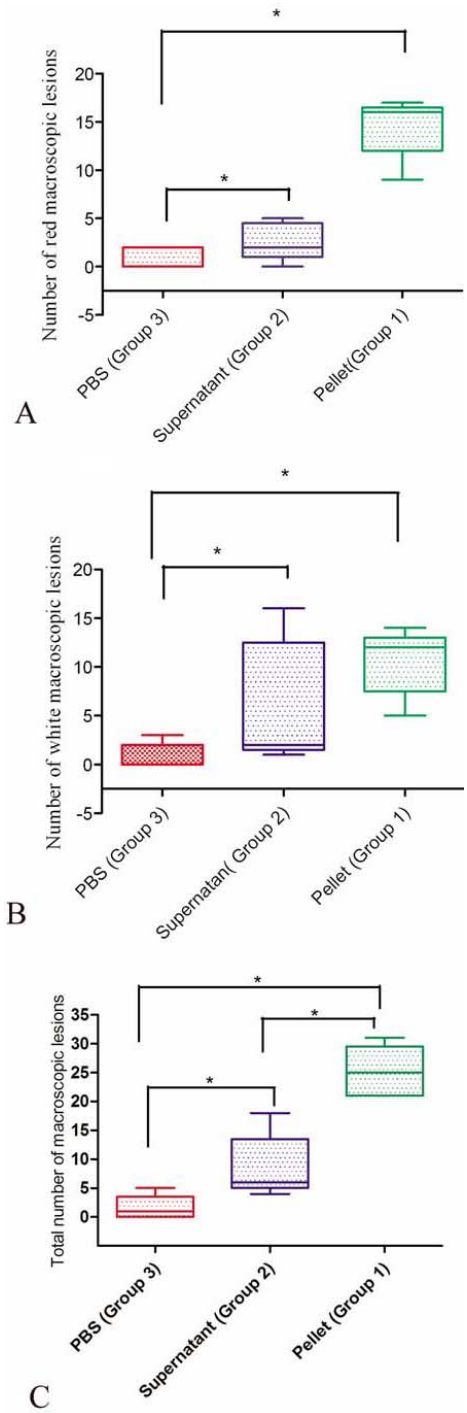

Figure 3. Higher number of red (A) and white (B) and total (C) lesions 25 days after induction with endometrial pellet when compared to induction with endometrial supernatant. The few lesions observed in the PBS group were all histologically negative for endometriosis. *P < 0.05

We speculate that the supernatant fraction of menstrual endometrium harbours molecules that are capable to induce the development of stromal or glandular peritoneal endometriosis in peritoneum, in view of the small amount of endometrial cells observed in menstrual supernatant. The exact nature of these inducing factors is still elusive and needs further investigation. Hepatocyte growth factor is a possible candidate as it has been described to be increased in PF from women with endometriosis compared to controls, to be present in endometriotic stromal cells, to induce critical changes in the morphology of mesothelial cells and to enhance

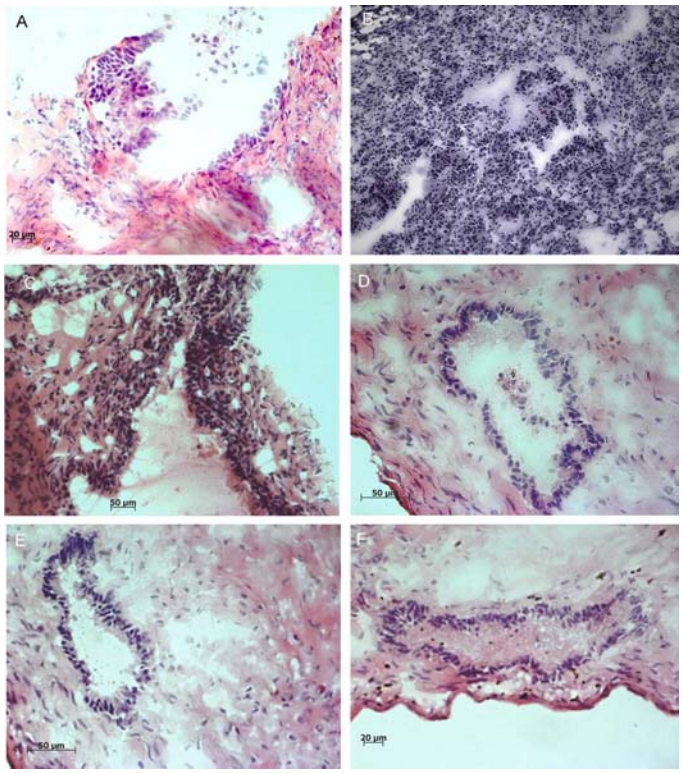

Figure 4. Histological appearance (haematoxylin and eosin) of biopsies obtained 25 days after induction of endometriosis using endometrial supernatant (A, B) or endometrial pellet (C, D, E & F) Peritoneal endometrial-like glands, possibly mullerian type epithelium. Stromal endometriosis without glandular component (X400).Typical endometriosis: dilated glandular epithelial structure surrounded by stromal cells. D, E & F Dilated peritoneal endometrial-like glands lined by columnar epithelium .

endometrial cell attachment and invasion (29). It is also likely that both menstrual phase endometrium and menstrual phase peritoneum are involved in this process, as we previously described that both menstrual endometrium and normal macroscopic peritoneum obtained during the menstrual phase contain more inflammatory cytokines, growth factors, adhesion factors and metalloproteinases in women with endometriosis when compared to women with a normal pelvis. (30;31;32). Women with endometriosis are known to have substantial alterations in their peritoneal microenvironment (33;34) that precede disease development or support the disease process including overtly enhanced angiogenesis (35), increased levels of inflammatory cytokines and macrophages (36;37).

The interpretation of our data in support of the induction theory is marked by several limitations. Firstly, it cannot be excluded that endometrial cells present in the endometrial supernatant were still the major factor leading to the development of endometriosis lesions by endometrial-peritoneal attachement and implantation. Indeed, we reported the presence of some endometrial cells in the supernatant fraction of menstrual endometrium obtained from 4 baboons in an additional experiment, using exactly the same protocol as in the main study. In future research, this limitation can be overcome by intrapelvic injection of the filtered cell free fraction of endometrial supernatant fraction, or injection of the cell free supernatant of cultured endometrial cells. Secondly, all baboons had at least one menstrual cycle before induction, raising the possibility that retrograde menstruation (38) was a cofactor in the development of endometriosis lesions. However, this limitation seems to be less important since no microscopic endometriosis was identified in serially sectioned biopsies in the control group (PBS) from our study, which had also been exposed to at least one cycle of menstruation. Nevertheless, this limitation can only be overcome by studying baboons that have had tubal sterilization before their first menstruation.

Based on data from our current study and previous studies (15;39;40), the injection of whole menstrual endometrium, a combination of endometrial menstrual cells and endometrial menstrual supernatant, is more successful (100% induction rate) for the induction of endometriosis than intrapelvic injection of the endometrial menstrual pellet without supernatant (at most 66% in our study, see Results section), which was in turn more successful than intrapelvic injection of menstrual endometrial supernatant including a much lower amount of endometrial cells (at most 33% in our study, see Results section). We hypothesize that both endometrial menstrual cells and menstrual fluid are essential components for the development of endometriosis. Although endometriosis was more efficiently induced (higher number and larger surface area of macroscopic lesions) by intrapelvic

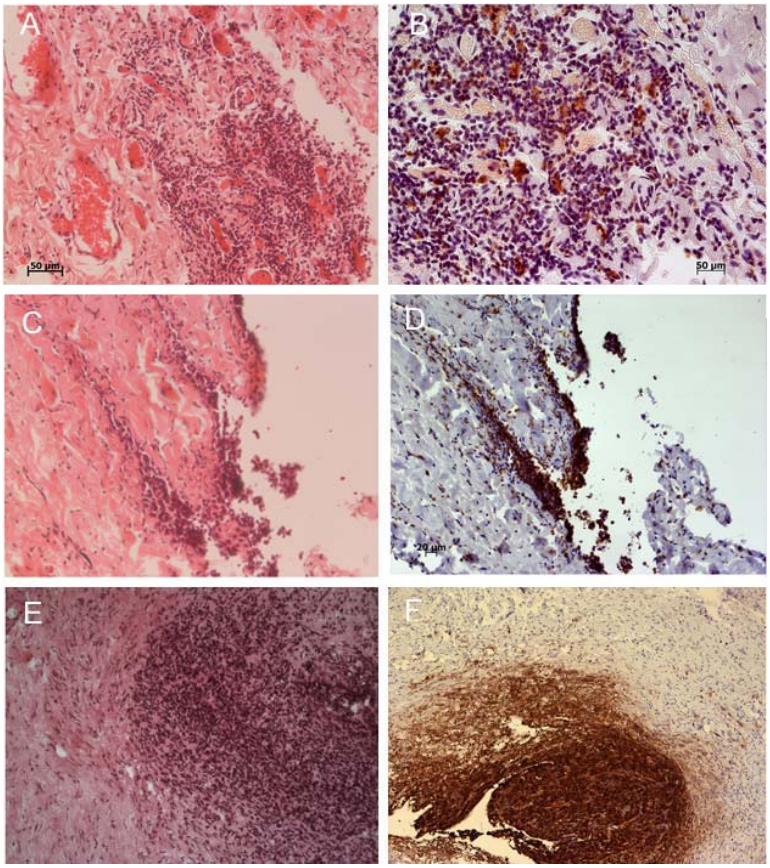

Figure 5. Combined histological (haematoxylin and eosin (A, C & E (X200)) and immunohistological (endometrial stromal cell marker CD 10 (B, D & F (X200)) appearance of biopsies obtained on day 1 after induction of endometriosis using endometrial supernatant (A-D) and on day 25 after induction with endometrial pellet (E-F).

injection of menstrual endometrial pellet than after intrapelvic injection of the menstrual endometrial supernatant, the prevalence of typical endometriosis containing both glands and stroma was much lower (7%) in biopsies after intrapelvic injection of menstrual endometrial pellet in the current study than observed within 25 days after intrapelvic injection of whole menstrual endometrium (15;39;40;41). An alternative explanation is the possibility that intrapelvic injection of separated menstrual endometrial pellet or menstrual endometrial supernatant leads predominantly to stromal endometriosis, observed as early as 1 day after induction, and that the histogenesis of typical endometriosis requires more than 25 days in both groups. These data provide a scientific basis to integrate the induction theory into the Sampson hypothesis that endometriosis is caused by regurgitation and subsequent peritoneal implantation of endometrial cells during retrograde menstruation (2). This theory is supported by substantial clinical, epidemiological and experimental evidence, as reviewed before (42). Indeed, various *in vitro* and *in vivo* studies have also shown that endometrial cells can adhere to and infiltrate the peritoneal surface (7;43;44; 45;46).

The histogenesis of stromal endometriosis can be explained by adhesion/infiltration in the endometrial pellet group and by induction of peritoneal metaplasia or by adhesion of endometrial cells in the endometrial supernatant group. *In vitro* human studies have shown that endometrium can adhere to autologous peritoneum *in vitro* within 2 days (7). Our *in vivo* baboon data revealed infiltration of CD10 positive endometrial stromal cells in the peritoneal surface within 24 hours after induction in both endometrial pellet and supernatant groups. The reported absence of CD10 staining in some biopsies that were histologically strongly suggestive for stromal endometriosis, can be explained by the observation that inflammatory cells co-exist with endometrial stromal cells in endometriosis lesions suggesting an inflammatory infiltration process (23).This explanation is in line with our previous data showing that both menstruation and intrapelvic injection of menstrual endometrium are associated with intrapelvic inflammation (47).Stromal endometriosis is a well described entity (20;21;23;48), present in up to 45% of endometriosis biopsies (48), characterized by the absence of endometriotic glands and the presence of microscopic nodules or plaques of endometrioid-type stroma, sometimes with a whorled pattern and prominent vascularity and erythrocyte extravasation (48). Stromal endometriosis can be found in close proximity with the surface mesothelium suggesting that stromal endometriosis may arise from mesothelial or

submesothelial fibroblast cells via a metaplastic process (48;49). It is also possible that endometrial stromal cells arise from local conversion of mesothelial cells as a result of epithelial to mesenchymal transition, a process behind the development of peritoneal fibrosis in patients on long term peritoneal dialysis (50). Emerging evidence suggests that epithelial-mesenchymal transitions represent an important source of mesenchymal cells involved in tissue repair and pathological processes (51). Inflammation and epigenetics may be key inducers especially in organ fibrosis and cancer progression (52). Epithelial-mesenchymal transition involving fibrosis is associated with inflammation, with many types of molecules produced by inflammatory cells and by resident activated fibroblasts (myofibroblasts). These molecules are reported to disrupt epithelial layers through degrading the basement membrane; consequently epithelial cells lose polarity and either undergo apoptosis or epithelial-mesenchymal transition (51;52).

The histogenesis of peritoneal endometrial-like glands without surrounding stroma containing a dilated glandular structure lined by columnar epithelium (suggestive for mullerian type epithelium) observed after intrapelvic injection of either endometrial pellet or endometrial supernatant requires further investigation. When only endometrial glandular epithelium is present, it is frequently difficult to accurately diagnose endometriosis, because this finding could represent endosalpingiosis, a usually asymptomatic microscopic finding (20;22). Endosalpingiosis refers to the presence of tubal-type glands (although if the ciliated cells are few in number, the gland could look like an endometrioid gland) that are thought to arise by a process of metaplasia from the peritoneum (20;22). The peritoneal endometrial-like glands observed may be the consequence of endometrial adhesion (pellet group), induction of peritoneal metaplasia (supernatant group, pellet group), mesenchymal to epithelial transition, but can also be remnants of the Mullerian system. In some cases of ovarian endometriomas, the diagnosis of endometriosis can be strongly suspected in the presence of endometrioid type glands together with hemorrhage in the form of pigmented-laden macrophages (20;48). In these cases, glandular epithelium is present whereas endometrioid type stroma is present only focally or is absent because of repeated hemorrhage and associated fibrosis.

In this study, it was not possible to test the hypothesis that stromal endometriosis and peritoneal endometrial-like gland may just be a transient change, which may disappear spontaneously, or may represent an early form of endometriosis which later develops into typical lesions marked by the presence of both endometrium and glands. In fact, it would be very difficult to design such a study, since it would require to take multiple biopsies of the same endometriotic lesion over time. Indeed, this can be considered to be practically impossible since peritoneal lesions in baboons are too small and since the process of taking a partial biopsy of a specific lesion may directly affect the future immunobiology and histological development of such a lesion.

Remodeling of spontaneous endometriosis lesion, defined by transition between typical, subtle and suspicious implants, has been reported in baboons during longitudinal follow-up studies up till 36 months (53, 54), and suggests that endometriosis in baboons is a dynamic and moderately progressive disease with periods of development and regression and with active remodelling between different types of lesions (42). This concept of remodelling was shown for the first time in baboons (53) and afterwards confirmed in women with endometriosis (55). In this study, we also observed that some lesions disappeared, emerged, or remodeled between Day 6 and Day 25, even though the total number of endometriotic red or white lesions and their endometriosis surface area were comparable on day 6 and day 25 after induction in all groups. However, the exact quantification of remodelling was not within the scope of this study.

In conclusion, we confirmed our hypothesis that endometriosis can be induced in baboons more successfully by intrapelvic injection of the pellet of menstrual endometrium when compared to intrapelvic injection of its supernatant. After intrapelvic injection of menstrual supernatant, the induction of endometriosis was likely caused by a few endometrial cells, or by soluble substances present in the endometrial supernatant, as has been proposed in the induction theory for endometriosis. Based on this study and on the results of previous studies, we hypothesize that both endometrial menstrual cells and menstrual fluid are essential components for the development of endometriosis.

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7. REFERENCES

1.David Adamson: Endometriosis: traditional perspectives, current evidence and future possibilities. *Int J Fertil Womens Med* 46, 51-68 (2001)

2.John A. Sampson. Peritoneal endometriosis due to menstrual dissemination of endometrial tissue into the peritoneal cavity. *Am J Obstet Gynecol* 14, 422-69 (1927)

3.William C. Keettel, Robert J. Stein. The viability of the cast-off menstrual endometrium. *Am. J. Obstet. Gynecol* 61,440-442 (1951)

4.John H. Ridley, Keith I. Edwards. Experimental endometriosis in the human. *Am. J. Obstet. Gynecol* 76, 783-790 (1958)

5.Sophie Debrock, Joseph A. Hill, Thomas M. D'Hooghe. Quantitative assessment of endometrial-peritoneal interaction in vitro: a non-invasive diagnostic test for women with endometriosis? *Gynecol Obstet Invest* 57,49-51 (2004)

6.Attila Bokor**,** Sophie Debrock**,** Maria Drijkoningen**,** Willy Goossens**,** Vilmos Fülöp, Thomas D'Hooghe. Quantity and quality of retrograde menstruation: a case control study. *Reprod Biol Endocrinol* 7, 123 (2009)

7.Sophie Debrock, Sarah Vander Perre, Christel Meuleman, Philippe Moerman, Joseph A. Hill, Thomas M. D'Hooghe. In-vitro adhesion of endometrium to autologous peritoneal membranes: effect of the cycle phase and the stage of endometriosis. *Hum Reprod* 17, 2523-2528 (2002)

8.Gustav Levander , P Normann. The pathogenesis of endometriosis. An experimental study. *Acta Obstet Gynecol Scand* 34, 366-398 (1955)

9.James A. Merrill. Endometrial induction of endometriosis across Millipore filters. *Am J Obstet Gynecol* 94, 780-790 (1966)

10.Nakamura M, Hidetaka Katabuchi, Toshimitsu Tohya, Yukitoshi Fukumatsu, Kohei Matsuura, Hitoshi Okamura. Scanning electron microscopic and immunohistochemical studies of pelvic endometriosis. *Hum Reprod* 8, 2218-2226 (1993)

11.NS Iwanoff. Drusiges cysthaltiges uterusfibromyom compliciert durch sacrom und carcinom (adenofibromyom cysticum sarcomatodes carcinomatosum)*Monatsschr Geburtsh Gynakol* 7, 295 – 300 (1898)

12.Robert Meyer. Uber eine adenomatose wucherung der serosa in einer bauchnarbe. *Zeitshr Geburtsh Gynak* 49, 32 – 41 (1903)

13.Kohei Matsuura, Hideyuki Ohtake, Hidetaka Katabuchi, Hitoshi Okamura. Coelomic metaplasia theory of endometriosis: evidence from *in vivo* studies and an *in vitro* experimental model. *Gynecol Obstet Invest* 47, 18-20 (1999)

14.Carolien A.M. Koks, Ayşe Y. Demir Weusten, Patrick G. Groothuis, Gerard A.J. Dunselman, Anton F.P.M. de Goeij, Johannes L.H. Evers. Menstruum induces changes in mesothelial cell morphology. *Gynecol Obstet Invest* 50, 13-8 (2000)

15.Thomas M. D'Hooghe, Charanjit S. Bambra, Barbara M. Raeymaekers, Inge De Jonge, Jozef M. Lauweryns, Philippe R. Koninckx. Intrapelvic injection of menstrual endometrium causes endometriosis in baboons (Papio cynocephalus, Papio anubis) *Am J Obstet Gynecol* 173, 125-134 (1995)

16.Thomas M. D'Hooghe, Charanjit S. Bambra, Freddy J. Cornillie, Mohamed Isahakia, Philippe R. Koninckx. Prevalence and laparoscopic appearance of spontaneous endometriosis in the baboon (Papio anubis- Papio cynocephalus) *Biol Reprod* 45,411-416 (1991)

17.American Fertility Society: Revised American Fertility Society Classification of Endometriosis. *Fertil Steril* 43, 351-2 (1985)

18.Revised American Society for reproductive Medicine classification of endometriosis: 1996. *Fertil Steril* 67, 817-821 (1997)

19.David B. Redwine. 'Invisible' microscopic endometriosis: a review. *Gynecol Obstet Invest* 55, 63-67 (2003)

20.Philip B. Clement. The pathology of endometriosis: a survey of the many faces of a common disease emphasizing diagnostic pitfalls and unusual and newly appreciated aspects. *Adv Anat Pathol* 14, 241-60 (2007)

21.Philip B. Clement, Robert H. Young. Two previously unemphasized features of endometriosis: micronodular stromal endometriosis and endometriosis with stromal elastosis. *Int J Surg Pathol* 8, 223-227 (2000)

22.Oisin Houghton, Glenn W. McCluggage. Pitfalls in the diagnosis of endometriosis: a condition characterized by a plethora of unusual histological features. In: Mini Symposium: Ovarian Pathology . Edit Glenn McCluggage. *Diagnostic Histopathology* 17**,** 193-202 (2011)

23.Clariss Potlog-Nahari, Andrew L. Feldman, Pamela Stratton, Deloris E. Koziol, James Segars, Maria J. Merino, Lynnette K. Nieman. CD10 immunohistochemical staining enhances the histological detection of endometriosis. *Fertil Steril* 82, 86-92 (2004)

24.Vaiyapuri P. Sumathi, Glenn W.McCluggage. CD10 is useful in demonstrating endometrial stroma at ectopic sites and in confirming a diagnosis of endometriosis. *J Clin Pathol* 55,391-2 (2002)

25.Shingo Fujii. Secondary mullerian system and endometriosis. *Am J Obstet Gynecol* 165,219-25 (1991)

26.Tadayuki Ishimaru, Khaleque Newaz Khan, Akira Fujishita, Michio Kitajima, Hideaki Masuzaki. Hepatocyte growth factor may be involved in cellular changes to the peritoneal mesothelium adjacent to pelvic endometriosis. *Fertil Steril* 81,810-8(2004)

27.Junichi Sugawara, Takao Fukaya, Takashi Murakami, Hidemune Yoshida, and Akira Yajima. Hepatocyte growth factor stimulates proliferation, migration, and lumen formation of human endometrial epithelial cells in vitro. *Biol Reprod* 57, 936–42 (1997)

28.Masakazu Yashiro, Yong-Suk Chung, Tohru Inoue, Shigehiko Nishimura, Tasuku Matsuoka, Tomofumi Fujihara and Michio Sowa. Hepatocyte growth factor

(HGF) produced by peritoneal fibroblasts may effect mesothelial cell morphology and promote peritoneal dissemination. *Int J Cancer* 67, 289–93 (1996)

29.Souichi Yoshida, Tasuku Harada, Masahiro Mitsunari, Tomio Iwabe, Yasuko Sakamoto, Satoru Tsukihara, Yumiko Iba, Sayako Horie, Naoki Terakawa. Hepatocyte growth factor/Met system promotes endometrial and endometriotic stromal cell invasion via autocrine and paracrine pathways. *J Clin Endocrinol Metab* 89, 823-32 (2004)

30.Cleophas M. Kyama, Attila Mihalyi, Peter Simsa, Henrik Falconer, Vilmos Fulop, Jason M. Mwenda, Karen Peeraer, Carla Tomassetti, Christel Meuleman, Thomas M. D'Hooghe. Role of cytokines in the endometrial-peritoneal cross-talk and development of endometriosis. *Front in Biosci (Elite Ed)* 1,444-454 (2009)

31.Cleophas M. Kyama, Lutgart Overbergh, Sophie Debrock, Dirk Valckx, Sarah Vander Perre, Christel Meuleman, Attila Mihalyi, Jason M. Mwenda, Chantal Mathieu, Thomas M. D'Hooghe. Increased peritoneal and endometrial gene expression of biologically relevant cytokines and growth factors during menstrual phase in women with endometriosis. *Fertil Steril* 85, 1667–75 (2006)

32.Cleophas M. Kyama, Lutgart Overbergh, Attila Mihalyi, Christel Meuleman, Jason M. Mwenda, Chantal Mathieu, Thomas M. D'Hooghe. Endometrial and peritoneal mRNA expression of aromatase, cytokines and adhesion factors in women with endometriosis. *Fertil Steril* 89, 301-310 (2008)

33.Janne Halme, Charles White, Scott Kauma, Joanne Estes, Stephen Haskill. Peritoneal macrophages from patients with endometriosis release growth factor activity in vitro. *J Clin Endocrinol Metab* 66, 1044–1049 (1988)

34.Jacques W. Ramey, David F.Archer. Peritoneal fluid: its relevance to the development of endometriosis. *Fertil Steril* 60,1-14 (1993)

35.Patrick G. Groothuis, Annemiek W. Nap, Ruth Grummer, Elke Winterhager. Vascular development in endometriosis. *Angiogenesis* 8, 47-56 (2005)

36.Yulia S. Antsiferova, Natalia Yu Sotnikova, Lyubov V. Posiseeva, Artur L. Shor. Changes in the T-helper cytokine profile and in lymphocyte activation at the systemic and local levels in women with endometriosis. *Fertil Steril* 84, 1705-11 (2005)

37.Khaleque Newaz Khan, Hideaki Masuzaki, Michio Kitajima, Ichiro Sekine, Tadayuki Ishimaru. Differential macrophage infiltration in early and advanced endometriosis and adjacent peritoneum. *Fertil Steril* 81,652-61 (2004)

38.Thomas M. D'Hooghe, Charanjit S. Bambra, Barbara M. Raeymaekers, Philippe R. Koninckx. Increased incidence and recurrence of retrograde menstruation in baboons with spontaneous endometriosis. *Hum Reprod* 11, 2022-2025 (1996)

39.Henrik Falconer, Jason M. Mwenda, Daniel C. Chai, Carrie Wagner, Attila Mihalyi, Peter Simsa, Cleophas Kyama, Freddy J. Cornillie, Agneta Bergqvist, Gabriel Fried, Thomas M. D'Hooghe. Treatment with Anti-TNF Antibody c5N reduces the extent of induced endometriosis in the baboon. *Hum Reprod* 21, 1856 -1862 (2006)

40.Dan I. Lebovic, Jason M. Mwenda, Daniel C. Chai, Michael D. Mueller, Allessandro Santi, Senait Fisseha, Thomas D'Hooghe. PPAR-gamma receptor ligand induces regression of endometrial explants in baboons: a prospective, randomized, placebo- and drug-controlled study. *Fertil Steril* 88,1108-19 (2007)

41.Thomas M. D'Hooghe, Noreen P. Nugent, Silvio Cuneo, Daniel C. Chai, Frank Deer, Sophie Debrock, Cleophas M. Kyama, Attila Mihalyi, Jason M. Mwenda. Recombinant human TNFRSF1A (r-hTBP1) inhibits the development of endometriosis in baboons: a prospective, randomized, placebo- and drug- controlled study. *Biol Reprod* 74, 131-136 (2006)

42.Thomas M. D'Hooghe: Clinical relevance of the baboon as a model for the study of endometriosis. *Fertil Steril* 68, 613-625 (1997)

43.Craig A. Witz, Iris A. Monotoya-Rodriguez, Robert S. Schenken. Whole peritoneal explants: a novel model of the early endometriosis lesion. *Fertil Steril* 71, 56-60 (1999)

44.Daisuke Aoki, Yukio Katsuki, Atsushi Shimizu, Chihaya Kakinuma, Shiro Nozawa. Successful heterotransplantation of human endometrium in SCID mice. *Obstet Gynecol* 83,220–8 (1994)

45.Jason S. Griffith, Ya-Guang Liu, Rajeshwar R. Tekmal, Peter A. Binkley, Alan E.Holden, Robert S. Schenken. Menstrual endometrial cells from women with endometriosis demonstrate increased adherence to peritoneal cells and increased expression of CD44 splice variants. *Fertil Steril* 93, 1745-9 (2010)

46.Michelle Nisolle, Francoise Casanas-Roux, Jacques Donnez. Early-stage endometriosis: adhesion and growth of human menstrual endometrium in nude mice. *Fertil Steril* 74, 306 –312 (2000)

47.Thomas M. D'Hooghe, Charanjit S. Bambra, Barbara M. Raeymaekers, Joseph A. Hill. Pelvic inflammation induced by diagnostic laparoscopy in baboons. *Fertil Steril* 72, 1134-1141 (1999)

48.David Boyle, W Glenn McCluggage. Peritoneal stromal endometriosis: A detailed morphological analysis of a large series of cases of a common and under-recognised form of endometriosis. *J Clin Pathol* 62,:530-3 (2009)

49.John W Bolen, Samuel P Hammar, Michael A McNutt. Reactive and neoplastic serosal tissue. A light-microscopic,

ultrastructural, and immunocytochemical study. *Am J Surg Pathol* 10, 34-47 (1986)

50.Luiz S. Aroeira, Abelardo Aguilera, José A. Sánchez-Tomero, Auxiliadora M. Bajo, Gloria del Peso, José A. Jiménez-Heffernan, Rafael Selgas, Manuel López-Cabrera. Epithelial to mesenchymal transition and peritoneal membrane failure in peritoneal dialysis patients: pathologic significance and potential therapeutic interventions. *J Am Soc Nephrol* 18, 2004-2013 (2007)

51.Raghu Kalluri, Eric G. Neilson. Epithelial-mesenchymal transition and its implications for fibrosis. *J Clin Invest* 112,1776-84 (2003)

52.Raghu Kalluri. EMT: When epithelial cells decide to become mesenchymal-like cells. *J. Clin. Invest* 119, 1417-1419 (2009)

Abbreviations: PF: Peritoneal Fluid; PBS: Phosphate Buffered Saline; AFS: American Fertility Society; ASRM: American Society for Reproductive Medicine ; TBS: Tris-buffered saline

Key Words: Typical Endometriosis, Stromal Endometriosis, Menstrual Endometrial Supernatant, Menstrual Endometrial Pellet, Baboons; White And Red Lesions

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