Establishment of an Endometriosis Model Using Cynomolgus Monkeys

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Establishment of an Endometriosis Model Using Cynomolgus Monkeys

A Dissertation Submitted to
the Graduate School of Life and Environmental Sciences,
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Abstract

Endometriosis is a common gynecological disease with an incidence of 10% in women aged 20 to 40 years. The main symptoms of endometriosis are pelvic pain and infertility. Currently, the only available pharmacological treatments are analgesics and hormones, whereas surgical treatment has a high recurrence rate. Therefore, new pharmacological treatments with different mechanisms are strongly needed. The etiology of endometriosis is not clear; however, it is suggested that reflux of menstrual bleeding triggers inflammation in the peritoneal cavity. Endometriosis spontaneously occurs only in few primates with cyclic menstrual bleeding that might trigger inflammation. One limitation for the development of new drugs for endometriosis is the lack of its testing in animal models that develop fibrotic lesions. In this study, I investigated whether spontaneous endometriosis in cynomolgus monkeys could reproduce the onset and progression mechanism of human endometriosis, and then verified the validity of the developed animal model.

In chapter I, I performed a macroscopic analysis, including assessing lesion and adhesion sites, and a histopathological analysis to study the role of inflammation in endometriosis. For this study, I used daily observational records, anatomical records, and pathological blocks of cynomolgus monkeys that were diagnosed with spontaneous endometriosis at the time of dissection. The macroscopic analysis revealed nodular lesions, cystic lesions and adhesions in 5, 7, and 8 out of 8 monkeys, respectively. Ovarian chocolate cysts, the typical clinical lesions, were observed with the progression to adhesion to the colon, uterus, etc. The histopathological analysis showed that in all 8 monkeys, endometriosis lesions were composed of 3 layers similar to those in humans.

The analysis of inflammation in endometriosis showed bleeding scars, immune cells, iron-stained hemosiderin macrophages, and CD31-positive angiogenic lesions in all 8 monkeys. In addition, it has been recently reported that endometriosis pain might be related to the development of ectopic nerve fibers in the lesions in humans. In this study, the expression of nerve fibre (NF), a marker of nerve fibers, was confirmed in 6 out of 8 animals.

In chapter II, I performed screening tests, quantitative laparoscopic stage analysis, and time course analysis by magnetic resonance imaging (MRI) and laparoscopy; in addition, I defined an exploratory endpoint related to endometriosis, and measured cytokine levels in the plasma. First, 29 monkeys were selected as candidate animals from approximately 600 female monkeys at the reproductive age at the Tsukuba Primate Research Center, based on the medical examination results. Fifteen of the 29 monkeys were confirmed to have endometriosis by laparotomy or laparoscopic examination. Although there was no screening method for monkeys that do not complain of pain, I established a screening method for endometriosis based on the findings of high CA125, abnormal abdominal palpation, abnormalities in fecal properties among monkeys with regular menstrual bleeding at the reproductive age. Then, laparoscopy was performed on 9 of the 15 monkeys with confirmed diagnosis, and lesions and adhesions were observed at the sites that are commonly affected in humans. I also examined whether the "Revised American Fertility Society Score (r-AFS score)" used in clinical practice could be used for monkeys with modifications, and showed its feasibility to quantitatively evaluate the disease status. Next, laparoscopic examination confirmed endometriosis in 8 out of the 9 monkeys, which were examined over time using laparoscopy to assign r-AFS scores and MRI to assess the lesion volume. The pathological condition showed a stable tendency.

In addition, I explored factors that fluctuated with menstruation, such as endometriosis pain and food intake. Monkeys with endometriosis exhibited reduced food consumption, compared to that of healthy monkeys, particularly during the menstrual cycle. Food consumption during menstruation tended to correlate with the adhesion scores (R^2 = 0.8239). Furthermore, I measured the plasma levels of 29 cytokines. Interleukin (IL)-1β levels were higher in monkeys with endometriosis than those in healthy monkeys; in addition, monocyte chemotactic protein 1 (MCP-1 or CCL2) and "regulated on activation, normal T cell expressed and secreted" (RANTES or CCL5) were higher in monkeys with severe endometriosis (stage III/IV) than those in healthy monkeys.

Finally, I characterized this endometriosis cynomolgus monkey model, compared to an established endometriosis model in baboons. Although baboons are easier for laparoscopic observation owing to their larger abdominal cavity, laparoscopy can also be performed for cynomolgus monkeys to quantitatively evaluate the disease status using the modified r-AFS score. In addition, no screening methods, MRI, or food consumption evaluation were performed for the endometriosis baboon model. In addition, securing a breeding space for cynomolgus monkeys is easy, and a whole genome analysis of cynomolgus monkeys is available in the literature.

In conclusion, this study revealed that spontaneous endometriosis in cynomolgus monkeys was macroscopically and pathologically similar to human endometriosis, and could be screened by combining blood tests and general routine examinations. It could be quantitatively evaluated over time using laparoscopy and MRI, where endometriosis was stable in most monkeys. These results suggested that this model satisfied the necessary conditions of an animal model. This model could be used to evaluate biological markers linked to endometriosis and measure the levels of inflammatory cytokines that are highly

expressed in human endometriosis. In the future, this model might be also used to elucidate the molecular mechanisms of fibrosis and adhesion progression, which is difficult with the conventional animal models, and to evaluate the efficacy of new drugs, particularly non-hormone drugs.

Abbreviations

CCL	CC chemokine ligand
CXCL	CXC chemokine ligand
EGF	epidermal growth factor
EpCAM	epithelial cell adhesion molecule
FGF	fibroblast growth factor
G-CSF	granulocyte-colony stimulating factor
GM-CSF	granulocyte macrophage colony-stimulating factor
HE	hematoxylin and eosin
HGF	hepatocyte growth factor
IFN	interferon
IL	interleukin
IP	interferon γ-induced protein
MRI	magnetic resonance imaging
NHP	non-human primates
QOL	quality of life
r-AFS score	revised-American Fertility Society score
TNF	tumor necrosis factor
MCP	monocyte chemotactic protein
MDC	macrophage-derived chemokine
MIF	macrophage migration inhibitory factor
MIG	monokine induced by interferon-γ
MIP	macrophage inflammatory proteins

I-TAC	interferon-inducible T cell-α chemoattractant
RANTES	regulated on activation, normal T cell expressed and secreted
VEGF	vascular endothelial growth factor

General introduction

Endometriosis is defined as the presence of endometrial glands and stroma outside the uterine cavity. It is an estrogen-dependent chronic inflammatory condition, which affects 2–10% of women during the reproductive age (Dunselman et al., 2014; Vercellini et al., 2014). The main symptoms of endometriosis are severe dysmenorrhea, chronic pelvic pain, bowel symptoms, and infertility (Greene et al., 2009). Currently, the only available pharmacological treatments are analgesics and hormones, whereas surgical treatment has a high recurrence rate. In addition, the current therapies cannot be used for long-term management of endometriosis. Therefore, new pharmacological treatments with different mechanisms are strongly needed.

The etiology of endometriosis is unclear; however, retrograde menstruation has been hypothesized to largely contribute to the disease pathogenesis. It has been suggested that endometrial fragments become attached to and invade the mesothelium, where a suboptimal immune response does not adequately clear the implants, resulting in their continued survival, growth, and cyclic bleeding, which in turn causes fibrosis and adhesion of several organs (Laux-Biehlmann et al., 2015). Thus, I suggested that endometriosis in monkeys with repetitive reflux of bleeding might reproduce the features of endometriosis in humans. Histopathological analysis of human endometriosis showed that ectopic nerve fibers and smooth muscle metaplasia were commonly observed in the fibrotic interstitium surrounding the epithelium (Odagiri et al., 2007; Zhang et al., 2016). One limitation for the development of new drugs for endometriosis is the lack of its testing in animal models that develop fibrotic lesions (Groothuis and Guo, 2018), since fibrosis is a pathological hallmark of endometriosis (Vigano et al., 2018).

One of the challenges in endometriosis research is to find an animal model with clinically relevant features and environment, including fibrosis with repetitive bleeding and inflammation. Spontaneous endometriosis in non-human primates (NHP) is useful for studying the disease because the anatomy of NHP reproductive organs is similar to that of humans (Braundmeier and Fazleabas, 2009; Story and Kennedy, 2004; Yamanaka et al., 2012). Although the menstrual cycle in rhesus monkeys is seasonal, the menstrual cycle in baboons (*Papio*) and cynomolgus monkeys (*Macaca fascicularis*) is similar to that in humans (around 4 weeks). Therefore, spontaneous endometriosis is considered relevant and was previously reported in cynomolgus monkeys (Ami et al., 1993; Fanton and Hubbard, 1983) and baboons (D'Hooghe et al., 1991; Merrill, 1968).

The histological characteristics of endometriosis in baboons have been fully described and are considered clinically relevant (D'Hooghe et al., 2009). Baboons are reportedly useful for endometriosis research because they are larger than cynomolgus monkeys, making it easier to evaluate the disease by laparoscopy and to obtain tissue samples. However, being smaller and a common laboratory animal, cynomolgus monkeys are easier to control in the laboratory setting.

In this study, I investigated whether spontaneous endometriosis in cynomolgus monkeys could reproduce the onset and progression mechanism of human endometriosis, and then verified the validity of the developed animal model. In chapter I, I performed a macroscopic analysis, including assessing lesion and adhesion sites, and a histopathological analysis to study the role of inflammation in endometriosis. In chapter II, I performed screening tests, quantitative laparoscopic stage analysis, and time course analysis by magnetic resonance imaging (MRI) and laparoscopy; in addition, I defined an exploratory endpoint related to endometriosis, and measured cytokine levels in the plasma.

Finally, this cynomolgus monkey model was characterized in comparison with the established endometriosis model in baboons.

Chapter I: Clinical relevance of endometriosis in cynomolgus monkeys

1. Introduction

Invasion, immune dysfunction, localized bleeding, and fibrosis are related to pain, which is the main symptom of endometriosis and a leading cause of the deterioration in the patient's quality of life (De Graaff et al., 2015). However, there is evidence that other mechanisms are also likely to contribute to the pain associated with endometriosis. These mechanisms may explain why the extent and morphological characteristics of the disease correlate poorly with the intensity and characteristics of pain in endometriosis (Asante and Taylor, 2011). Additional mechanisms underlying endometriosis pain include smooth muscle metaplasia and innervation of the diseased tissue (Barcena de Arellano et al., 2011b; Barcena de Arellano and Mechsner, 2014; Morotti et al., 2014; Odagiri et al., 2009).

The underlying pathophysiology of endometriosis is far from being well understood; thus, animal models are crucial for further research. Because of the anatomical similarity with the human reproductive organs, non-human primates (NHP) are considered candidate model organisms to study endometriosis (Braundmeier and Fazleabas, 2009; Story and Kennedy, 2004; Yamanaka et al., 2012). There have been some reports of spontaneous endometriosis in baboons (Cornillie et al., 1992; Dehoux et al., 2011; Dick et al., 2003), rhesus monkeys (Ito et al., 2001) and cynomolgus monkeys (Ami et al., 1993; Cline et al., 2008; Fanton and Hubbard, 1983). Among those, baboons are commonly used to study endometriosis because they are larger, making it easier to obtain

tissue samples using laparoscopy and to perform complex surgeries. (Braundmeier and Fazleabas, 2009; D'Hooghe et al., 2009). However, because baboons are scarce, and research facilities that can handle animals of their size are limited, the more widely used experimental animals, such as rhesus and cynomolgus monkeys, might be more convenient than baboons (Yamanaka et al., 2012). Since the menstrual cycle in cynomolgus monkeys is continuous over 4 weeks, similar to that in humans, whereas that of rhesus monkeys is seasonal, cynomolgus monkeys might be preferable in terms of the clinical relevance (Yamanaka et al., 2012).

A previous study on endometriosis in cynomolgus monkeys of the breeding colony (Ami et al., 1993) showed that the prevalence and basic histopathological characteristics were largely similar to those previously reported (Cline et al., 2008; Fanton and Hubbard, 1983). However, the involvement of smooth muscle metaplasia and lesion innervation in endometriosis in cynomolgus monkeys has not been investigated. In addition, several recent clinical reports have described endometriotic lesions in the lymph nodes and discussed their clinical importance as a potential cause of disease recurrence in women receiving surgical treatment of endometriosis (Mechsner et al., 2010; Tempfer et al., 2011). Therefore, I provide here a detailed pathological characterization of histologically confirmed spontaneous endometriosis in cynomolgus monkeys from a large breeding colony.

2. Materials and methods

2.1. Animals

The study was conducted on cynomolgus monkeys from the breeding colony of Tsukuba Primate Research Center (TPRC) at the National Institute of Biomedical

Innovation, Health and Nutrition (Ibaraki, Japan). Five animals were sacrificed by exsanguination under deep anesthesia with ketamine hydrochloride (Ketalar, Daiichi Sankyo Propharma Co., Ltd., Tokyo, Japan) and xylazine hydrochloride (Seractal, Bayer Yakuhin, Ltd., Osaka, Japan) followed by barbiturate administration (pentobarbital sodium, Somnopentyl®, Kyoritsu Seiyaku Co., Ltd., Tokyo, Japan). The other three monkeys were found dead. In the necropsy of these 8 animals, lesions that were grossly diagnosed as endometriotic were used for the study.

Six monkeys were bred in the laboratory, and the other 2 were from Cambodia. All animals were reared at the primate center and were housed indoors in individual cages. The age of all monkeys ranged between 5 to 21 years. Four monkeys had experienced pregnancy and giving birth (of which 2 experienced cesarean section), and the other 4 had never experienced pregnancy (Table 1-1). Menstruation of all animals was periodically observed.

The animals were kept at 25 ± 2°C with 60 ± 5% relative humidity, and a 12-h light-dark cycle. Animals were provided with water *ad libitum* and fed daily with 70 g of commercially prepared monkey chow (35 pieces of type AS, Oriental Yeast, Tokyo, Japan) and 100 g of apple.

2.2. Ethical approval

All experimental monkeys in this study were cared for using procedures approved by the Animal Care and Use Committee of the National Institutes of Biomedical Innovation, Health and Nutrition. Besides, protocols for all experiments involving animals were in compliance with the guidelines set by the Institute for the care, use, and biological hazard countermeasures of laboratory animals.

2.3. Histopathology

After gross observation, each lesion was dissected from the animals, fixed in 10% neutral buffered formalin. Paraffin-embedded thin sections were stained with hematoxylin and eosin (HE), according to the conventional method. Diagnosis of adenomyosis was made if endometrial glands and stromal cells were observed in the inner 90% of the myometrium of the uterine wall (Bergeron et al., 2006).

The representative sections were also stained for iron by Berlin blue dye (2% potassium ferrocyanide solution; Muto Pure Chemicals, Tokyo, Japan). In addition, some sections were used for immunohistochemical detection of CD10, a marker of stromal cells (McCluggage et al., 2001), CD31, a marker of blood vessels (Witmer et al., 2002), α-smooth muscle actin (SMA) and PGP9.5, a marker of nerve fibers (Morotti et al., 2014).

Briefly, for immunohistochemistry, after antigen retrieval by a microwave, the sections were incubated with primary antibodies against CD10 (1:80; clone: 56C6, AbD Serotec, Oxford, UK), CD31 (2 μg/mL; clone: JC70A, Dako, Glostrup, Denmark), α-SMA (1 μg/mL; clone: 1A4, Dako, Glostrup, Denmark), and PGP9.5 (2.0 μg/mL; rabbit polyclonal, Dako, Glostrup, Denmark) at 4°C overnight. Each primary antibody was detected with EnVision™+ (Dako, Glostrup, Denmark), visualized with diaminobenzidine (Wako Pure Chemical, Osaka, Japan), and counterstained with hematoxylin (Muto Pure Chemicals, Tokyo, Japan). Then, they were examined using a light microscope (Optiphot-2, Nikon, Tokyo, Japan).

3. Results

3.1. General components of the endometriotic lesions in cynomolgus monkeys

The gross examination of the 8 cynomolgus monkeys with endometriotic revealed adhesions between several pelvic and abdominal organs in all animals, and chocolate cysts were observed in 4 out of 8 animals. Cysts, including blueberry cysts, and nodules were also observed in all animals (Fig. 1-1, Table 1-2).

Table 1-3 lists the microscopic findings in monkeys with endometriosis. The walls of the cystic lesions, including the chocolate cysts, were composed of endometrial and stromal cells, where the latter was confirmed by CD10 immunohistochemistry (Fig. 1-2A, B). In addition, hemorrhage in the cysts and/or inflammatory cells within the cyst or cyst wall was often accompanied with hemosiderin-laden macrophages (Fig. 1-2C, D) that were positive for iron staining (Berlin blue dye, Fig. 1-2E). Copious blood vessels were also observed (Fig. 1-2F).

In 3 monkeys, adenomyosis was observed in the inner 90% of the myometrium (Table 1-3). The lesions were composed of endometriotic endometrial and stromal cells surrounded by reactive hypertrophic myometrium, and the endometriotic cells exhibited similar morphology to that observed during the proliferative stage of the menstrual cycle (Fig. 1-3A, B). All of those lesions were accompanied with similar lesions in the outer 10% of the myometrium or parametrium (Fig. 1-3C). In addition, tissue blocks of the Douglas pouch showed some lesions that invaded the colonic muscle layer and reached the submucosa (Fig. 1-3D-F).

3.2. Smooth muscle metaplasia, interstitial fibrosis, and innervation of the endometriotic lesions in cynomolgus monkeys

Cells stained with α-SMA were observed in all monkeys (Table 1-3). These cells were identical to those reported in patients (namely interstitial smooth muscle cells) that surrounded the endometriotic epithelium and stromal components (Fig. 1-4A). In other parts, endometriotic epithelium and stromal components were surrounded with a thick and tight layer of interstitial connective tissue, and in some cases, the stromal components could no longer be observed. In tissue sections showing adhesions, interstitial fibrosis constituted the main component of the section. In addition, PGP9.5 immunohistochemistry revealed sporadic distribution of unmyelinated fine nerve fibers throughout the lesions (Fig. 1-4B).

3.3. Endometriotic lesions in the lymph nodes of cynomolgus monkeys

Endometriotic lesions within the lymph nodes were incidentally observed in 2 monkeys (Table 1-4) in tissue sections of the mesocolic border of the colon. Endometriotic epithelium and stromal components, which were confirmed with CD10 immunohistochemistry, were observed; however, no interstitial components, such as connective tissue or smooth muscles, were observed (Fig. 1-4C-E).

4. Discussion

Eight cynomolgus monkeys were diagnosed with spontaneous endometriosis, and their characteristics were compared with those of clinical endometriosis. The gross features (distribution and characteristics) of endometriosis in cynomolgus monkeys were similar to those of human endometriosis (Burney and Giudice, 2012; Giudice and Kao,

2004; Khan et al., 2008; Vercellini et al., 2014). Microscopically, endometriotic epithelial and stromal components were observed with copious blood supply and inflammation in monkeys with endometriosis. Since hemorrhage and hemosiderin-laden macrophages were also observed, local bleeding might have occurred during the menstrual cycle, similar to that in patients (Asante and Taylor, 2011; Burney and Giudice, 2012). Since iron staining revealed the presence of metal-overloaded macrophages, which might play a role in the suboptimal immune response in endometriosis (Pirdel and Pirdel, 2014), further studies are needed to investigate the relationship between metal overload and the pathophysiology of the disease.

In three monkeys, pelvic endometriosis was associated with adenomyosis. The distribution of the lesions and morphological characteristics of cells in the lesions were similar to those in patients with endometriosis (Bergeron et al., 2006). Since all monkeys showed endometriosis in the outer 10% of the myometrium or parametrium, they would be categorized as subtype II adenomyosis, which is hypothetically caused by an invasion of endometriosis from outside the uterus, resulting in disruption of the uterine serosa (Kishi et al., 2012). In addition, deep infiltrating endometriosis has been described in humans (Koninckx et al., 2012), and could be reproduced in some cynomolgus monkeys, which exhibited deeply invasive lesions in the colon. Therefore, I concluded that spontaneous endometriosis (and adenomyosis) in cynomolgus monkeys reproduced the basic clinical characteristics of the disease, which is in line with previous findings (Ami et al., 1993; Sato et al., 2012).

The endometriotic epithelium and stromal components were surrounded by α-SMA-positive smooth muscles, with similar distribution and organization to those of clinical endometriosis (Barcena de Arellano et al., 2011b). Smooth muscle metaplasia has

been proposed to originate from different cells. In addition, several studies suggested that the contraction of smooth muscles might contribute to endometriosis-related pain in patients (Barcena de Arellano et al., 2011b; Barcena de Arellano and Mechsner, 2014; Odagiri et al., 2009). Because of its identical distribution, spontaneous endometriosis in cynomolgus monkeys might offer a promising model for the understanding of the nature of smooth muscle metaplasia and its contribution to pelvic pain.

Very severe interstitial fibrosis was observed in all lesions in cynomolgus monkeys. This finding is considered clinically important because it often results in adhesions between the pelvic and abdominal organs, and thus may contribute to pain and infertility in patients (Barcena de Arellano et al., 2011a; Barcena de Arellano and Mechsner, 2014; Morotti et al., 2014; Odagiri et al., 2009). In addition, PGP9.5-positive unmyelinated nerve fibers were observed in the endometriotic lesions. Studies on rat and human endometriotic lesions demonstrated that sympathetic and sensory nerves developed within the lesions, and in the rat model, the fibers were directly connected to the central nervous system *via* the splanchnic and vagus nerves (Berkley et al., 2005). It has been suggested that sympathetic innervation of the metaplastic smooth muscles may stimulate muscle contraction, resulting in pelvic pain (Odagiri et al., 2009). Additionally, the sensory nerve might contribute to endometriosis-associated pain because several immune mediators in the lesions can directly stimulate this nerve (Laux-Biehlmann et al., 2015; Morotti et al., 2014; Odagiri et al., 2009). Therefore, it is crucial to find alternative therapies, other than surgery and medications that suspend the menstrual cycle, to manage endometriosis-associated pain. Drugs that prevent nerve excitation might be candidate therapies for endometriosis-related pain (Laux-Biehlmann et al., 2015; Morotti et al., 2014). Since nerve fibers were also observed in cynomolgus monkeys in this study,

monkey endometriosis might be a promising preclinical tool for developing drugs against nerve-related pain.

Additionally, the lymph nodes within endometriosis were found in two monkeys. The lesions were composed of an endometrial component and a CD10-positive stromal component, which is similar to clinical findings. It has been suggested that endometriosis in the lymph nodes may be related to recurrence after surgery or lymphatic dissemination of the disease (Mechsner et al., 2010; Tempfer et al., 2011). Gene expression profiling of lymphatic endometriosis revealed differential expression of several genes, including epithelial cell adhesion molecule (EpCAM), CDH1 (E-cadherin), CXCR4, and CD44, thus lymphatic endometriosis was considered to have its own biological nature (Burkle et al., 2013). Only small-scale clinical studies on lymphatic endometriosis have been approved because the clinical nature of the lesions is far from well understood. Therefore, it is crucial to improve the understanding of lymphatic endometriosis to enable large-scale clinical studies (Mechsner et al., 2010; Tempfer et al., 2011). Because one limitation of studying lymphatic endometriosis is that lymphatic lesions are incidentally found in cynomolgus monkeys, more thorough investigations of the monkey abdominal and pelvic lymph nodes are needed in the future provide information, such as correlation to the disease stage, biological characteristics of the cells and their responses to drugs, correlation to the recurrence rate after surgery, and evidence of lymphatic dissemination. This information might enable the implementation of large-scale clinical studies in the future.

The current investigation of spontaneous endometriosis in cynomolgus monkeys revealed that many aspects of clinical endometriosis were reproduced in this model. It is noteworthy that several disease components in cynomolgus monkeys might contribute to

endometriosis-related pain and disease recurrence. If laparoscopic observation over time and surgical manipulation of the lesions are feasible in cynomolgus monkeys, similar to that in baboons (Braundmeier and Fazleabas, 2009), I can further study the clinical implications of these components. If biomarkers that correlate with endometriosis-associated pain in monkeys can be found, further investigations of the newly proposed concepts will be possible. Therefore, I tried to use laparoscopy to monitor the lesions and to find potential biomarkers of endometriosis-related pain in cynomolgus monkeys in chapter II.

5. Tables

Table 1-1. Reproductive histories of the cynomolgus monkeys with histologically confirmed endometriosis.

Animal no.	Age at necropsy (years)	Total pregnancies (times mated)	Vaginal delivery	Caesarean sections	Abortions	Years since previous pregnancy at diagnosis	Menstruations during previous 6 months
00178	5.1	NA (0)	NA	NA	NA	NA	2
00180	5.8	NA (0)	NA	NA	NA	NA	5
93164	12.4	2 (14)	1	0	1	2.5	5
93144	13.9	1 (10)	1	0	0	4.5	3
94043	15.7	0 (11)	NA	NA	NA	NA	6
94014	16.6	NA (0)	NA	NA	NA	NA	3
89093	20.6	6 (32)	5	1	0	4.3	6
85005	21.1	12 (22)	9	3	0	1.7	7

NA, not applicable.

Table 1-2. Macroscopic findings in cynomolgus monkeys with histologically confirmed endometriosis.

Animal no.	Nodule	Cyst	Adhesion
00178	Co^1, Om^2, Ub, Ut		Co-Om, Om-Ub, Ub-Ut
00180	Co^1, Si, Ut^2, Om	Co^1, Ut^2, Si, Om	Co-Ut^3, Si-Ut, Ub-Ut^2, Pt
93164	Dp^1	Ut^2	Nodule-Pt, Ub-Ut
93144		Ch^1, Dp^2, Co, Om, Ut^2	Co-Ut, Om-Ut
94043		Ch^1	Om-Ut^2, Ub-Ut, Co-Ut, Si-Ut, St-Pc^3
94014		Ch^1, Pt^2	Ch-Pt
89093	Ut	Ch^1, Dp, Ut^2,	Om-Ub, Om-Ut, Ub-Ut
85005	Pt	Co^1, Ub, Ut^2	Co-Pt, Pt-Ub, Pt-Ut

[a]The slide number (1, 2 or 3) corresponding to the histopathological findings is shown in Table IV. Ch, chocolate cyst (large cyst that sometimes includes ovary, uterus and several pelvic organs); Dp, Pouch of Douglas; Co, colon; Om, omentum; Ov, ovary; Pt, peritoneal wall; Si, small intestine; Ub, urinary bladder; Ut, uterus; St, stomach; Pc, pancreas; empty columns, no finding.

Table 1-3. Histopathological findings in cynomolgus monkeys with histologically confirmed endometriosis.

Animal no.	Slide no. (organs)[a]	Findings[b]
00178	1 (Co)	Ep, Sc, Fi, He, Ic, Hm, Sm, Nf
	2 (Om)	Ep, Sc, Fi, He, Ic, Hm, Sm, Nf
00180	1 (Co)	Ep, Sc, Fi, He, Ic, Hm, Ly, Nf
	2 (Ut, Ub-Ut)	Ep, Sc, Fi, He, Ic, Hm, Sm, Nf
	3 (Co-Ut)	Ep, Sc, Fi, He, Ic, Hm, Sm, Nf
93164	1 (Dp)	Ep, Sc, Fi, He, Ic, Hm, Sm
	2 (Ut)	Ep, Sc, Fi, He, Ic, Hm, Ad, Sm
93144	1 (Ch)	Ep, Sc, Fi, He, Ic, Hm, Sm
	2 (Dp, Ut)	Ep, Sc, Fi, He, Ic, Hm, Sm, Nf
94043	1 (Ch)	Ep, Sc, Fi, He, Ic, Hm, Sm, Nf
	2 (Om-Ut)	Ep, Fi, He, Ic, Hm, Ad, Sm
	3 (St-Pc)	Ly
94014	1 (Ch)	Ep, Sc, Fi, He, Ic, Hm, Sm
	2 (Pt)	Ep, Sc, Fi, Ic, Hm, Sm
89093	1 (Ch)	Ep, Sc, Fi, He, Ic, Hm, Sm, Nf
	2 (Ut)	Ep, Sc, Fi, He, Ic, Hm, Sm
85005	1 (Co)	Ep, Sc, Fi, He, Ic, Hm, Sm, Nf
	2 (Ut)	Ep, Sc, Fi, He, Ic, Ad

[a]The slide number and organs correspond with those in macroscopic findings. Ch, chocolate cyst; Dp, Douglas pouch; Co, colon; Om, omentum; Pt, peritoneal wall; Ub, urinary bladder; Ut, uterus; St, stomach; Pc, pancreas.

[b]Microscopic findings: Ep, endometriotic epithelium; Sc, stromal cell; Fi, fibrotic interstitium; He, haemorrhage; Ic, inflammatory cells; Hm, hemosiderin-laden macrophages; Ad, adenomyosis; Ly, lymph node endometriosis; Sm, smooth muscle metaplasia; Nf, nerve fibre.

6. Figures

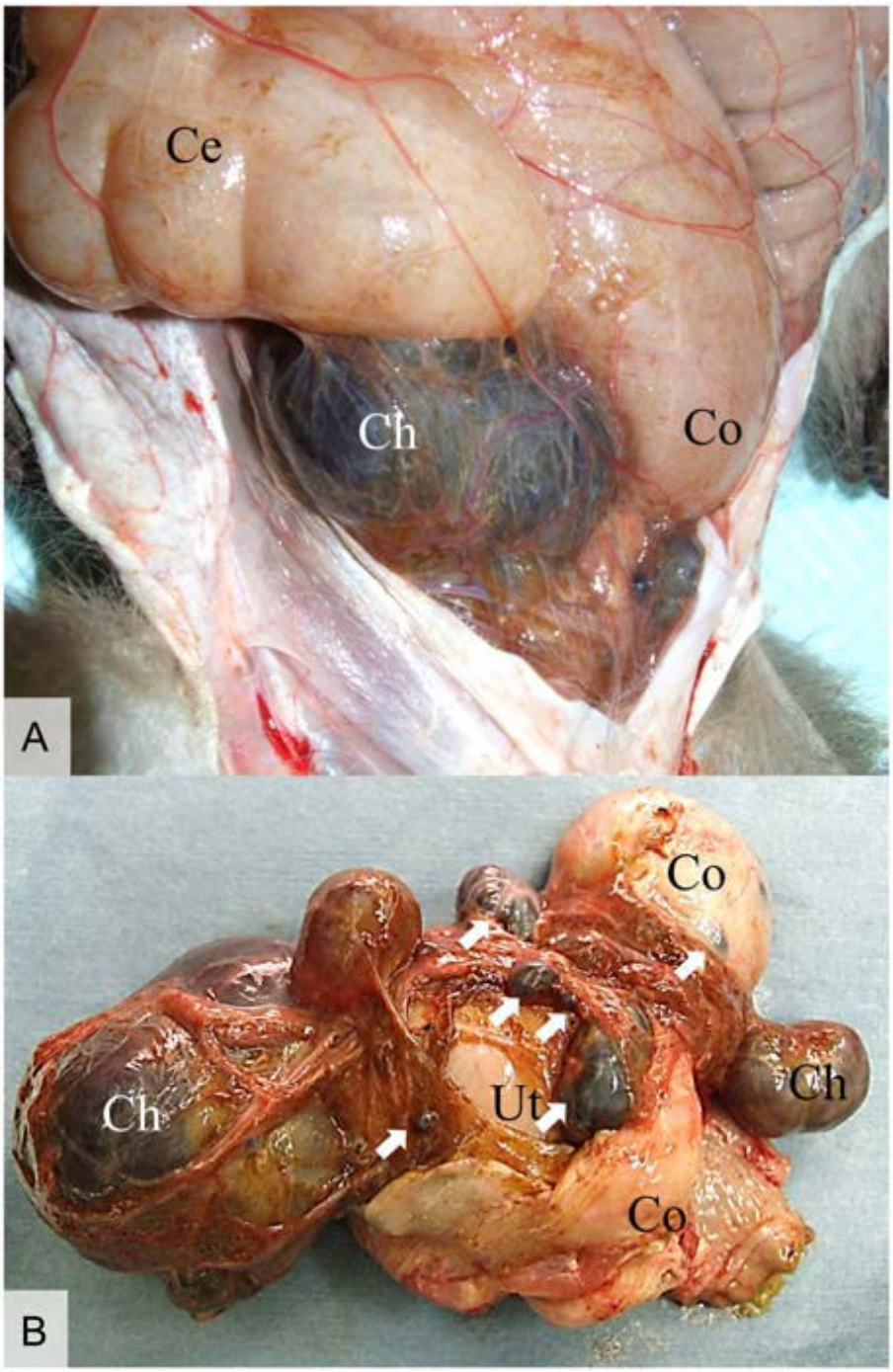


Figure 1-1. Gross appearance of spontaneous endometriosis in cynomolgus monkey. (A) Ventral aspect of the lesion of 93144. A large chocolate cyst (Ch) was observed, and the caecum (Ce) and colon (Co) were inflated with gas. (B) The same dissected lesion from the same animal. Uterus (Ut) was entangled with several organs by severe fibrosis. Arrows indicate small cysts or nodular lesions.

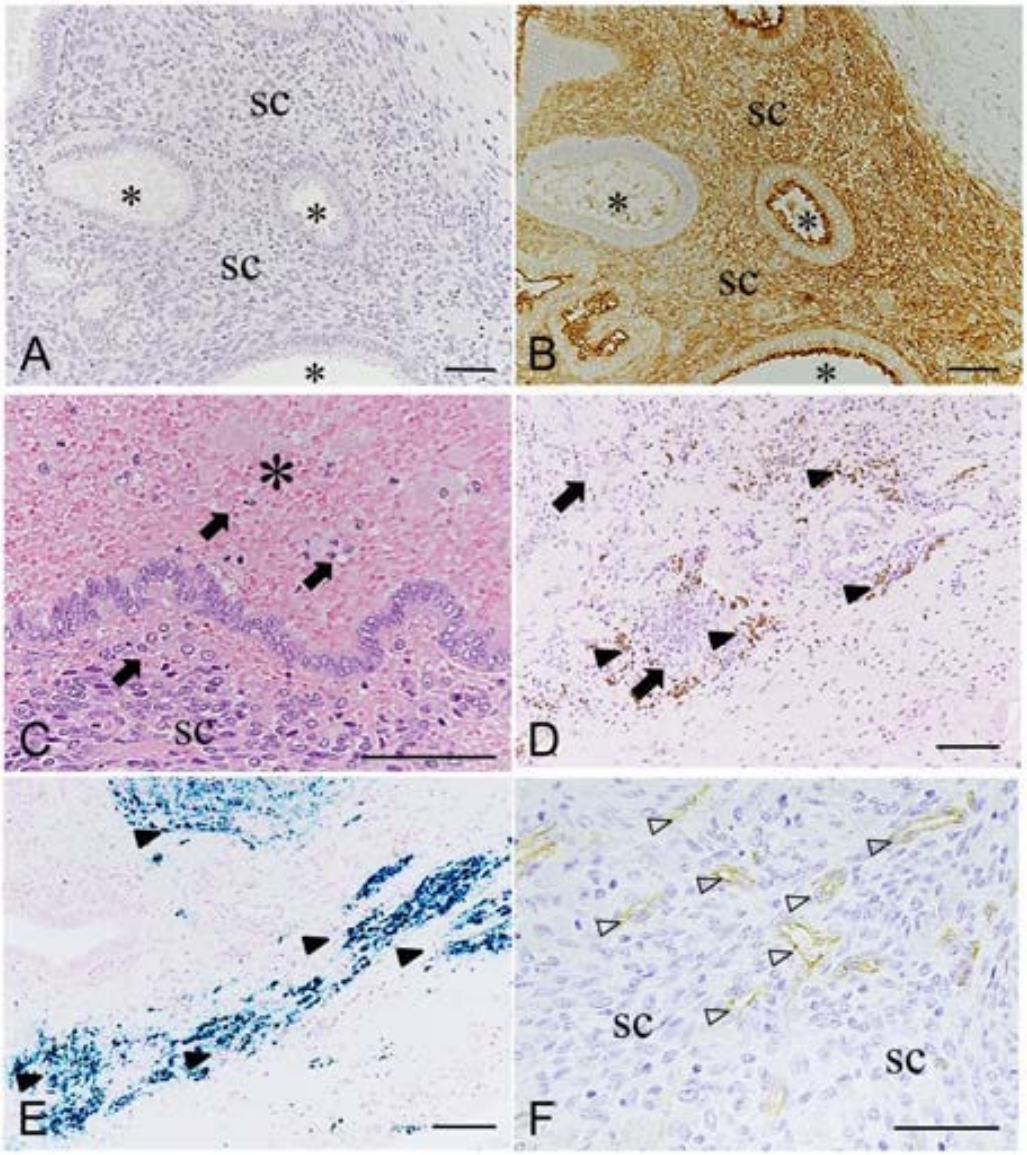


Figure 1-2. Basic components of endometriotic lesions in cynomolgus monkey. (A) The lumen of a cyst (asterisks) was covered with endometriotic epithelium and surrounded with stromal cells (sc). HE staining. Bar = 10 μm. (B) The stromal cells were positive for CD10. CD10 immunostaining of a serial section of (A). Bar = 10 μm. (C) The lumen of the cyst (asterisk) was filled with haemorrhage and inflammatory cells (arrows). HE staining. Bar = 10 μm. (D) Haemosiderin-laden macrophages (closed triangles) and inflammatory cells (arrows) were observed in the cyst wall. HE staining. Bar = 10 μm. (E) The macrophages were positive to iron staining (closed triangles). Berlin blue. Bar = 10 μm. (F) Ample CD31- positive blood vessels (open triangles) were observed, mainly in the stromal (sc) and interstitial areas. CD31 immunostaining. Bar = 5 μm.

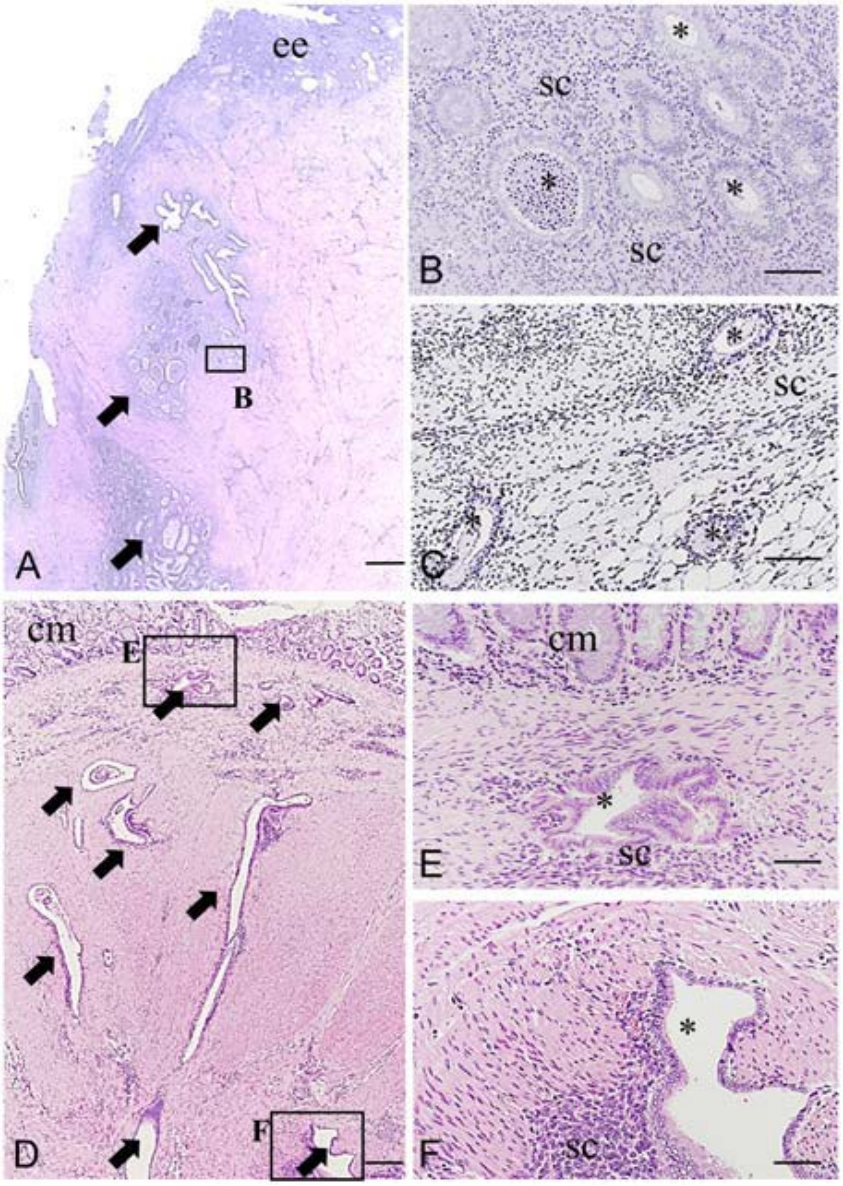


Figure 1-3. Adenomyosis and deep infiltrating endometriosis of the colon. (A) Lesions caused by adenomyosis (arrows) were distributed within the myometrium of the uterine wall. Eutopic endometrium (ee). HE staining. Bar = 1 mm. (B) Higher magnification of adenomyosis (box area in A). The lumen of the glands (asterisks) was covered with endometriotic epithelium and surrounded with stromal cells (sc). HE staining. Bar = 10 μm. (C) Similar lesions to those in (B) were also observed in the parametrium. HE staining. Bar = 10 μm. (D) The endometriotic lesions (arrows) invaded the colon muscle layer and reached through to the submucosa. Colonic mucosa (cm). HE staining. Bar = 50 μm. (E and F) Higher magnifications of boxes in D. The lumen of the glands (asterisks) was covered with endometriotic epithelium and surrounded with stromal cells (sc). Colonic mucosa (cm). HE staining. Bar = 10 μm.

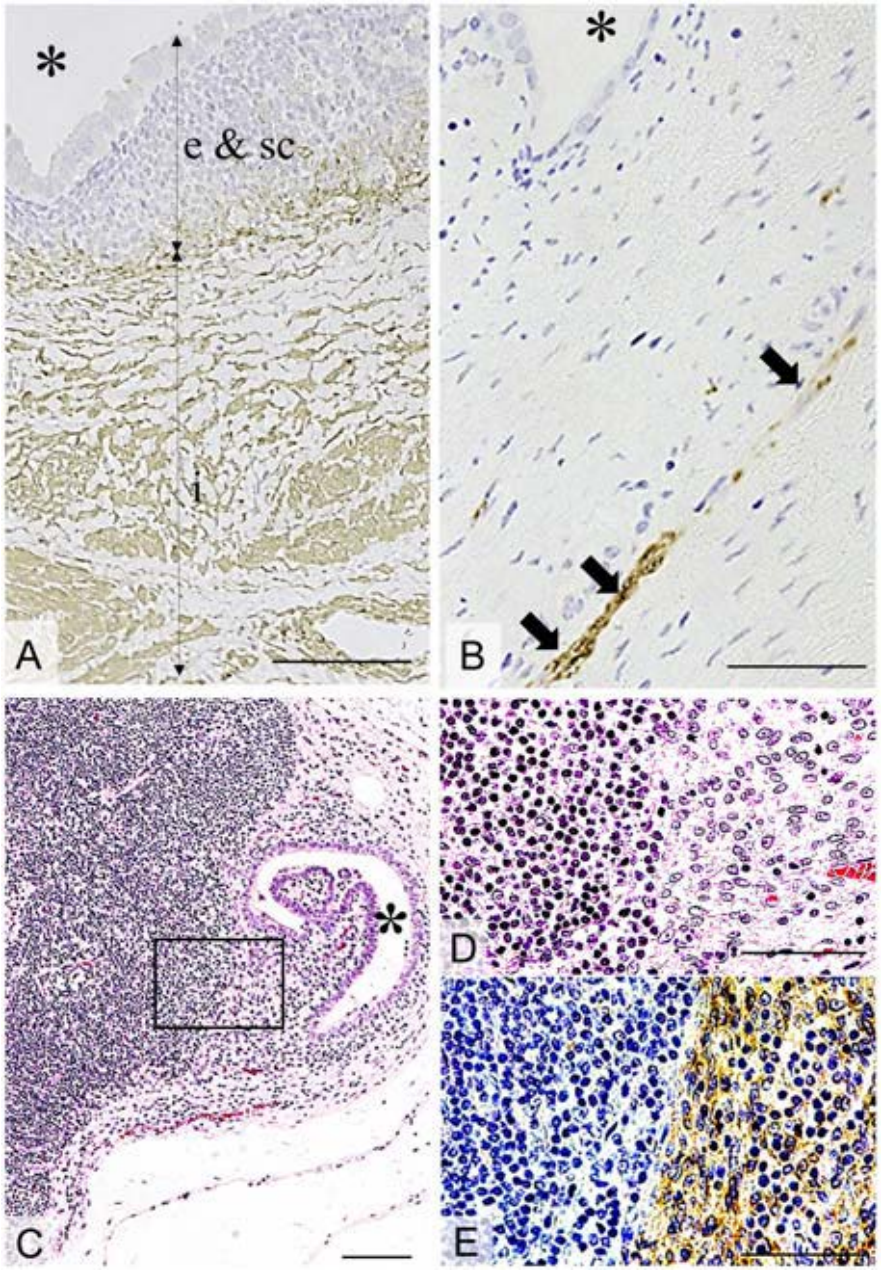


Figure 1-4. Smooth muscle metaplasia, nerve fibres and an endometriotic lesion in the lymph node. (A) The endometriotic epithelium and stromal cells (e & sc) were surrounded by metaplastic smooth muscle that was observed in the interstitium (i). α-SMA immunostaining. Bar = 10 μm. (B) PGP9.5-positive nerve fibres were observed (arrows). PGP9.5 immunostaining. Bar = 10 μm. (C) An endometriotic gland (asterisk) was observed in the lymph node. HE staining. Bar = 10 μm. (D) High magnification of the boxed area of C. HE staining. Bar = 5 μm. (E) CD10-positive stromal cells were observed in the serial sections of C and D. CD10 immunostaining. Bar = 5 μm.

Chapter II: The use of spontaneous endometriosis in cynomolgus monkeys as a clinically relevant experimental model

1. Introduction

For an animal model of spontaneous endometriosis to evaluate drug efficacy, it should fulfill three requirements; it should be suitable for screening, diagnosis with staging, and monitoring. Although D'Hooghe et al. (1992) showed that laparoscopy could be used for endometriosis diagnosis and monitoring with staging (over 30 months) in baboons (D'Hooghe et al., 1996a; D'Hooghe et al., 1996b), a systematic approach for screening for spontaneous endometriosis in NHPs from a general population has not been reported. In addition, although MRI monitoring has recently received increasing attention as a non-invasive method in clinical setting (Bazot et al., 2004; Takahashi et al., 2016), there are no reports of its use in baboons or cynomolgus monkeys. Because of the lack of an endometriosis model fulfilling these requirements, I tried to establish methods for screening, staging, and monitoring of spontaneous endometriosis in cynomolgus monkeys.

Since pain is the main symptom and endpoint in clinical endometriosis (Dunselman et al., 2014), parameters that correlate with endometriosis-associated pain in animals need to be established. For this purpose, in the current study, I investigated surrogate parameters that might correlate with endometriosis-associated pain.

Furthermore, I analyzed inflammation and immune cells that were suggested to be involved in endometriosis. Several studies showed that inflammatory mediators, such as

IL-8, MCP-1, RANTES, and IL-1β, were elevated in the plasma and peritoneal fluid of patients with endometriosis (Beste et al., 2014; Borrelli et al., 2015; Harada et al., 2001).

2. Materials and methods

2.1. Ethical approval

The study was conducted on cynomolgus monkeys from the breeding colony of Tsukuba Primate Research Center (TPRC) at the National Institute of Biomedical Innovation, Health and Nutrition (Ibaraki, Japan). All monkeys used in this study were cared for using procedures approved by the Animal Care and Use Committee of the National Institute of Biomedical Innovation, Health and Nutrition. Besides, protocols for all experiments involving animals were in compliance with the guidelines set by the Institute for the care, use, and biological hazard countermeasures of laboratory animals.

2.2. Animals

The study was conducted on approximately 600 female cynomolgus monkeys at the reproductive age in the breeding colony of TPRC from 2008 to 2012. Monkeys were kept at 25 ± 2°C with 60 ± 5% relative humidity and a 12 h light-dark cycle. Animals were provided with water *ad libitum* and fed daily with 70 g of commercially available solid food (35 pieces of type AS, Oriental Yeast Co. Ltd., Tokyo, Japan) and 100 g of apple.

During the screening phase (explained below), 29 monkeys were selected as potential candidates, 15 of which were diagnosed by laparoscopy and/or open surgery, 9 were categorized by laparoscopy according to the disease stage, and 8 of them were monitored for several months (Fig. 2-1).

Other 5 healthy female monkeys were selected arbitrarily and observed for 8 weeks as a control to measure the amount of food consumption.

Plasma samples were obtained from other 16 healthy female monkeys to measure the control levels of 29 cytokines.

2.3. Screening

Twenty nine monkeys (7 to 21 years old) were selected as potential candidates by screening of the monkeys, primarily according to the regularity of menstrual bleeding (5 times per year or more) and then based on the high values of serum CA125 (> 35 U/mL) and/or abnormal palpation during routine medical examination. Serum CA125 concentration was measured with HISCL CA125 reagent (Sysmex Co., Ltd., Kobe Japan) and K-4500 (Sysmex Co., Ltd., Kobe Japan). Typical palpation abnormalities included enlargement, induration, irregular shape of the uterus, and abdominal distension.

At TPRC, the general conditions of all monkeys were observed daily and recorded as follows: feces (normal, loose feces, diarrhea, no feces), amount of menstrual bleeding (no bleeding, spotting, mild bleeding, heavy bleeding), food consumption (most, half, little), and locomotor activity (normal, low activity, inactivity/lying down). In a health examination carried out every two years, hematological parameters and blood biochemistry, including C-reactive protein (CRP), are analyzed using K-4500 (Sysmex Co., Ltd., Kobe Japan), and the clinicopathological status is recorded. In addition, mating times, pregnancy, delivery, cesarean sections, and abortion are recorded.

To increase the number of monkeys with endometriosis, I investigated the screening parameters by retrospectively assessing the routine laboratory parameters recorded at the TPRC. These additional screening parameters and combinations of all screening

parameters were then evaluated according to the positive predictive rate (PPV) and “sensitivity”. The PPV and “sensitivity” are defined as:

PPV = true positive / (true positive + false positive)

Sensitivity = true positive / (true positive + false negative)

In addition, serum CA125 level was measured in endometriotic monkeys with or without chocolate cyst(s).

2.4. Diagnosis with staging

Fifteen out of 29 monkeys were definitively diagnosed with endometriosis by open surgery (n = 5) or laparoscopy (n = 10, one monkey was excluded from the study because of a gastric tumor). The biopsy samples were taken from all 5 monkeys diagnosed by open surgery and 3 out of the 9 monkeys diagnosed by laparoscopy. The samples were fixed with 4% (V/V) neutral buffered formalin and stained with HE. The disease status of the nine monkeys diagnosed by laparoscopy was evaluated based on the lesion size, location, and adhesion. It was not possible to have comparable staging data for the monkeys diagnosed by open surgery because the smaller lesions were sometimes hard to detect. Examination by open surgery and laparoscopy were both performed under anesthesia with ketamine hydrochloride (Daiichi Sankyo Propharma Co., Ltd., Tokyo Japan) and xylazine hydrochloride (Bayer AG, Leverkusen Germany) followed by barbiturate (Kyoritsu Seiyaku Co., Ltd., Tokyo Japan).

To evaluate the disease status in cynomolgus monkeys, the revised American Fertility Society Score (r-AFS score)(American Fertility Society, 1985) was modified in 2 ways (modified r-AFS score): 1) the size criteria were changed to 5 and 15 mm, 2) the item adhesion at vesicouterine pouch, such as the Douglas pouch, (complete, partial,

open) was added. When monkeys underwent laparoscopy twice during the monitoring period, the modified r-AFS score was used to evaluate the lesion size, location, and adhesion.

2.5. Disease monitoring

Disease monitoring is shown in the final step in the experimental scheme (Fig. 2-1). One out of the nine monkeys that were diagnosed by laparoscopy dropped out from the study and was used in another study. The disease status of the eight monkeys diagnosed with endometriosis was monitored by laparoscopy and MRI (SEMENS Healthcare Japan Inc., Tokyo Japan) for 2-7 months. The monitoring schedule of each animal is shown in Supplemental Fig. 2-1. T1-weighted images were captured to quantitatively evaluate the cysts. Since periodic analysis showed that the cyst volume was stable during all estrus phases (menstrual, proliferative, and secretory phases), the data of all time-points were comparable.

In the time course monitoring, I quantitatively evaluated the lesions by comparing the detectable lesion profile (size and location) detected by MRI with the laparoscopic findings.

2.6. Endometriosis-associated biological parameters

I previously reported that monkeys with histologically confirmed endometriosis showed a decrease in food consumption during menstruation (Nishimoto-Kakiuchi et al., 2016). Therefore, I selected food consumption as a candidate surrogate parameter for endometriosis-related pain. I quantitatively assessed the food consumption of the 8

monkeys for 12 weeks and analyzed its correlation with the modified r-AFS score at the second laparoscopy (Supplemental Fig. 2-1).

2.7. Measurement of inflammatory cytokine levels

The levels of 29 cytokines were measured using Cytokine 29-Plex Monkey Panel (Thermo Fisher Scientific, MA USA) with reactivity with cynomolgus and rhesus monkeys, such as IL-1β, IL-1RA, IL-2, IL-4, IL-5, IL-6, IL-10, IL-12, IL-15, IL-17, granulocyte-colony stimulating factor (G-CSF), granulocyte macrophage colony-stimulating factor (GM-CSF), interferon (IFN)-γ, interferon-γ induced protein (IP)-10 (CXCL10), tumor necrosis factor (TNF)-α, eotaxin (CCL11), IL-8 (CXCL8), MCP-1 (CCL5), macrophage-derived chemokine (MDC or CCL22), macrophage migration inhibitory factor (MIF), monokine induced by interferon-γ (MIG or CXCL9), macrophage inflammatory proteins (MIP)-1α (CCL3), MIP-1β (CCL4), Interferon-inducible T cell-α chemoattractant (I-TAC or CXCL11), RANTES(CCL5), epidermal growth factor (EGF), fibroblast growth factor (FGF)-basic, hepatocyte growth factor (HGF) and vascular endothelial growth factor (VEGF). I measured the levels of the 29 cytokines in monkeys with mild (stage I/II, n = 3) and severe (stage III/IV, n = 6) endometriosis, compared to those in healthy monkeys (n = 16).

2.8. Statistical analysis

The correlations between CA125 and the presence of chocolate cyst, as well as that between food consumption and the modified r-AFS scores of the second laparoscopy were analyzed using Wilcoxon rank-sum test. Each cytokines in monkeys with stage I/II and stage III/IV were compared to those in healthy monkeys using Dunnett’s test.

3. Results

3.1. Screening

Using laparoscopy or open surgery, I definitively diagnosed 15 out of 29 candidate monkeys, which were selected first by the existence of regular menstruation, then by the presence of abnormal palpation and/or serum CA125 > 35 U/mL. Endometriosis was confirmed in 8 out of 15 monkeys by histological examination of biopsy tissue.

Because the PPV of the first screening method (using a high level of CA125 and/or abnormal palpation) was 51.7% (15/29), I retrospectively analyzed general screening parameters in order to find a method with higher PPV for a more effective screening of endometriosis and with a higher sensitivity to reduce false negative findings (Fig. 2-2A). Palpation was a good parameter for the first screening because its sensitivity was 100% (15/15). I found that fecal examination might be a good screening tool because the PPV of fecal examination was high (83.3%; 5/6). Thus, a triple combination of abnormal palpation, high CA125 level, and fecal abnormality further increased the PPV to 76.9% (10/13). Although the sensitivity of no feces was low (33.3%; 5/15), the sensitivity of fecal monitoring was high (86.7%; 13/15) and that of the triple combination was moderate (66.7%; 10/15).

In another approach used after the screening phase, I followed the 9 monkeys that were not diagnosed with endometriosis. Endometriosis was diagnosed in 4 of these monkeys during the follow-up (Fig. 2-1). These findings suggested that monkeys with aberrant palpation, abnormal feces, and/or high CA125 level were possible candidates of developing endometriosis in the future, and intensive monitoring of the animals might be another efficient way to increase the sample size.

In addition, heavy menstrual bleeding had a high PPV (75%, 6/8) but low sensitivity at 40%, 6/15). CRP, locomotor activity, mating times, pregnancy, delivery, and cesarean sections did not correlate with any of disease aspects (data not shown). Moreover, serum CA125 levels tended to be higher in monkeys with chocolate cyst(s) than in those without chocolate cysts (Fig. 2-2B). This finding suggested that serum CA125 levels could be useful for diagnosing chocolate cysts; however, it might be less efficient for screening for peritoneal lesions without chocolate cysts.

3.2. Diagnosis with staging

Representative laparoscopic images are shown in Fig. 2-3. Full laparoscopic examination could detect small endometriotic lesions, including cystic/nodular/subtle lesions and adhesions, and to observe adhesions in the Douglas pouch, even if the body size of the female monkeys was relatively small (2.4–5 kg in this study). Additionally, small lesions in the peritoneal wall and large chocolate cysts were detectable (Fig. 2-3A, B) with the different colors (red, pink, brown, blue, black, and/or white). Adhesions were observed in the ovaries, fallopian tubes, Douglas pouch, and the vesicouterine pouch (Fig. 2-3C, D). It is noteworthy that lesions and adhesions at the vesicouterine pouch were frequent (44.4%; 4/9), which might be a characteristic of endometriosis in cynomolgus monkeys. Altogether, this information allowed us to calculate a modified r-AFS score for each animal and to assign its corresponding disease stage (stage I to IV) (Table 2-1).

3.3. Disease monitoring

Laparoscopic findings and the r-AFS scores were monitored at the indicated time-points for 2 to 7 months (Fig. 2-4, Supplemental Fig. 2-1). The disease status tended to be maintained, and sometimes disease progression was observed (Fig. 2-4B-D).

MRI examination showed that large cystic lesions could be monitored using T1-weighted images, which allowed us to detect cystic lesions behind adhesions and also in deep infiltrating endometriosis, which are difficult to monitor by laparoscopy. For example, MRI revealed that the chocolate cysts expanded into the serosa of the dorsal uterus in monkey number 89102 (Fig. 2-5A); however, laparoscopy could only distinguish chocolate cysts in monkeys (Fig. 2-5B). The total volume of the cystic lesions and the r-AFS score in each animal tended to be stable (Fig. 2-5C).

3.4. Endometriosis-associated biological parameters

I quantitatively assessed food consumption in the 8 monkeys with endometriosis, compared to that in healthy control monkeys. In healthy control animals, food consumption was considered 100% (median), whereas it decreased in monkeys with endometriosis and dropped even further during menstrual bleeding (Fig. 2-6A, B). I also found that food consumption during menstrual bleeding correlated with the adhesion r-AFS score (Fig. 2-6C) but not with the size r-AFS score (data not shown).

4. Discussion

There is a need to establish an endometriosis model to clarify the etiology of the disease and test potential therapies. Spontaneous endometriosis in NHPs is a promising

candidate model for that purpose. For an animal model of spontaneous endometriosis to evaluate drug efficacy, it should fulfill three requirements; it should be suitable for screening, diagnosis with staging, and monitoring.

To screen for cynomolgus monkeys with spontaneous endometriosis from the general population, I assessed several parameters obtained as a part of usual laboratory practice, and selected abdominal palpation abnormality, fecal examination, and high levels of serum CA125 as sufficient parameters. Gastrointestinal symptoms, such as diarrhea and constipation, were reported as major clinical predictive markers of endometriosis (Maroun et al., 2009). Thus, I first screened the monkeys for abnormal palpation. However, for a large sample size, it may be more efficient to select the animals with constipation or that are positive for the three screening parameters. Moreover, animals, in which the first diagnostic examination failed to detect an endometriotic lesion, could be pooled with animals with a single positive screening parameter and kept under intensive monitoring, which might further increase the detection rate.

In the search for screening parameters, I found that serum CA125 levels correlated with the presence of chocolate cysts in cynomolgus monkeys with endometriosis. Therefore, a high level of serum CA125 could be used in future studies as a potential predictor of in the presence of chocolate cysts.

The laparoscopic findings showed that spontaneous endometriosis in cynomolgus monkeys had various manifestations in terms of the location, size, and color of lesions, as well as the location and extension of adhesions. Because these manifestations were similar to the clinical symptoms and disease staging might be essential, I tried to optimize the clinical r-AFS score for cynomolgus monkey endometriosis. To modify the r-AFS score, first I changed the criteria for the lesion size (to 5 mm and 15 mm), because the

monkeys are smaller than humans. I also added the item of adhesion at the vesicouterine pouch besides the Douglas pouch (complete, partial, open) because adhesions at this pouch were more frequently observed in cynomolgus monkeys (44.4%; 4/9) than in humans (1.6-12%) (Chapron et al., 2010; Siesto et al., 2014). With those minor modifications, I could calculate the r-AFS score and assign the disease stage for each animal.

Disease monitoring by laparoscopy showed that the disease status was stable and sometimes progressed for several months (up to 7 months). In addition, since MRI monitoring has recently received increasing attention as a non-invasive method in the clinical setting (Bazot et al., 2004; Takahashi et al., 2016), MRI was used for monitoring of spontaneous endometriosis of cynomolgus monkeys. I showed that MRI could detect large cystic lesions and give information on the cyst volume; in addition, it could sometimes detect lesions that were undetectable using laparoscopy because the view was obstructed. Table 2-2 summarizes the individual merits and demerits of laparoscopy and MRI. In addition, a decrease in food consumption was observed during menstruation in monkeys with spontaneous endometriosis. In clinic, it is well known that women with pelvic endometriosis frequently report gastrointestinal complaints, such as catamenial painful defecation that increases in intensity during menstruation (Fauconnier et al., 2002). Furthermore, suppression of menses over several months led to a significant decrease in the intensity of digestive symptoms, such as rectal tenesmus and dyschezia (Fedele et al., 2001). Thus, I suggested that the decrease in food consumption in cynomolgus monkeys with spontaneous endometriosis could serve as a surrogate marker for clinical gastrointestinal complaints. Since the solid food was commercially available, data about

food consumption from other facilities might also be important to increase the validity of these findings.

5. Tables

Table 2-1. Modified revised American Fertility Society score and stage for each of the nine cynomolgus monkeys with spontaneous endometriosis.

Animal ID	95001	94123	92193	91001	93124	89074	96146	94203	89102
Size									
Peritoneum	0	I	I	0	0	2	0	0	4
Right ovary	0	0	0	16	20	20	4	20	NE
Left ovary	0	0	0	0	0	0	20	NE	0
Size score	0	I	I	16	20	22	24	20	4
Adhesions									
Right ovary	0	0	0	2	4	16	16	16	16
Left ovary	0	0	0	0	8	I	16	16	16
Right tube	0	0	0	0	0	0	0	16	16
Left tube	0	0	0	0	0	0	0	16	16
Pouch of Douglas	4	4	4	0	4	4	0	0	40
Vesicouterine pouch	0	0	4	0	4	0	4	NE	4
Adhesion score	4	4	8	2	20	21	36	64	108
Total score	4	5	9	18	40	43	60	84	112
Disease stage	I	I	II	III	III	IV	IV	IV	IV

NE, not examined.

Table 2-2. Comparison of characteristics of laparoscopy and magnetic resonance imaging.

Item	Laparoscopy	MRI
Diagnosis	Easily possible	Possible, especially for large lesions
Quantification of lesion size	Semi-quantification for any lesion	Possible for large cystic lesions
Qualification of the lesion	Location, color, depth, and extension of even small lesions and adhesions were evaluable	Location of large lesions was evaluable (even if the lesion was hidden behind an adhesion)
Staging	Possible with r-AFS score	Not possible
Invasiveness of the procedure	Relatively invasive	Non-invasive
Technical expertise	Higher skill is required	Skills for MRI operation are required

r-AFS, revised American Fertility Society.

6. Figures

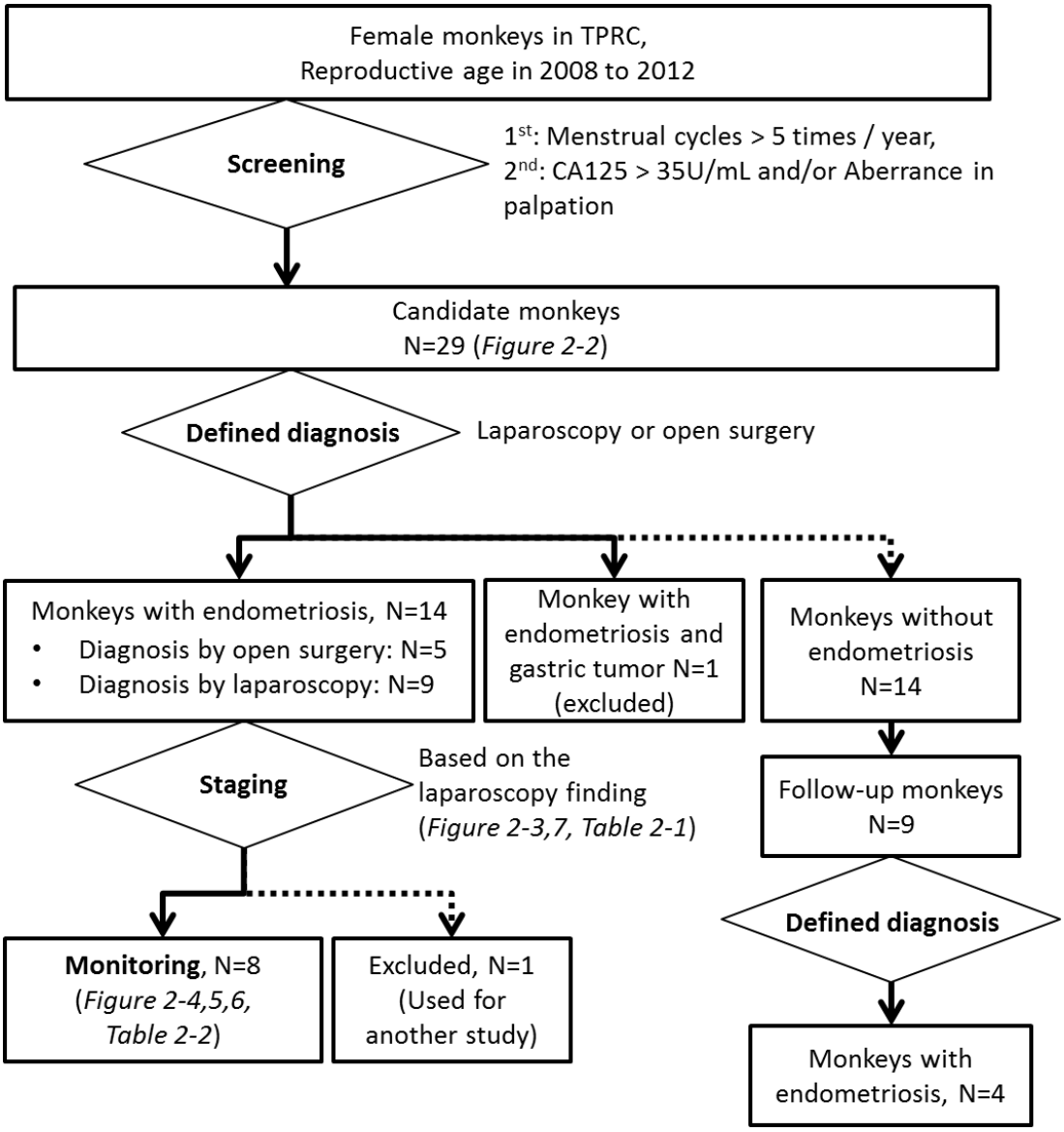


Figure 2-1. Summary of the experimental scheme for investigating spontaneous endometriosis in cynomolgus monkeys. TPRC, Tsukuba Primate Research Center.

(Modified from Figure 1, Nishimoto-Kakiuchi et al., 2018)

A

Screening items	# of positive animal	true positive	PPV (%)	Sensitivity (%)
Palpation	25	15	60.0	100.0
CA125	17	10	58.8	66.7
Feces	25	13	52.0	86.7
Diarrhea	14	7	50.0	46.7
Loose feces	22	11	50.0	73.3
No feces	6	5	83.3	33.3
Triple positive in palpation, CA125 and Feces	13	10	76.9	66.7

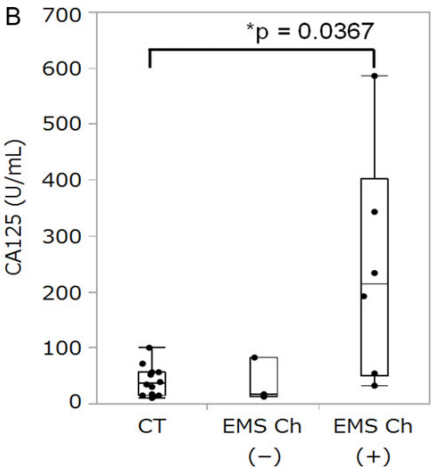


Figure 2-2. Retrospective assessment of items used for screening cynomolgus monkeys with endometriosis. (A) Heat map of the positive predictive value (PPV) and sensitivity for endometriosis of each screening item. (B) Relationship between CA125 and presence of chocolate cysts. Serum CA125 was significantly higher in endometriosis monkeys with chocolate cysts (n = 6) than in control non-endometriosis monkeys (n = 14) (P = 0.0367, Steel test), whereas in those without chocolate cysts (n = 3) it tended to be similar to the control monkeys. EMS, endometriosis.

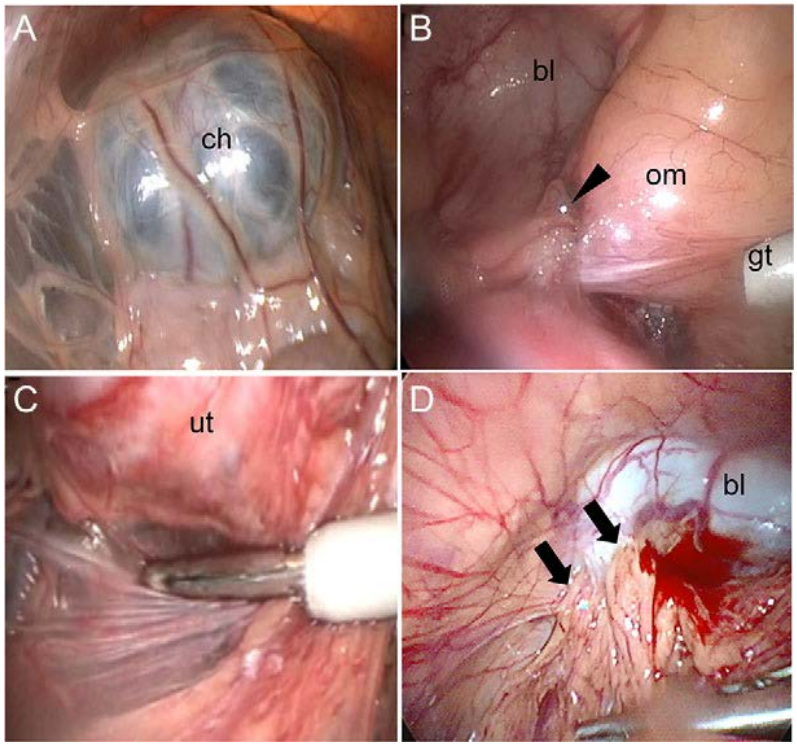


Figure 2-3. Representative laparoscopic images of endometriosis in cynomolgus monkeys. (A) Chocolate cyst (ch, 94203). (B) Small lesion (arrowhead) in the peritoneal cavity (92193). (C) Adhesion at the Douglas pouch (93124). (D) Adhesion (arrow) at the bladder (93124). ch, chocolate cyst; bl, bladder; om, omentum; ut, uterus; gt, guide tool.

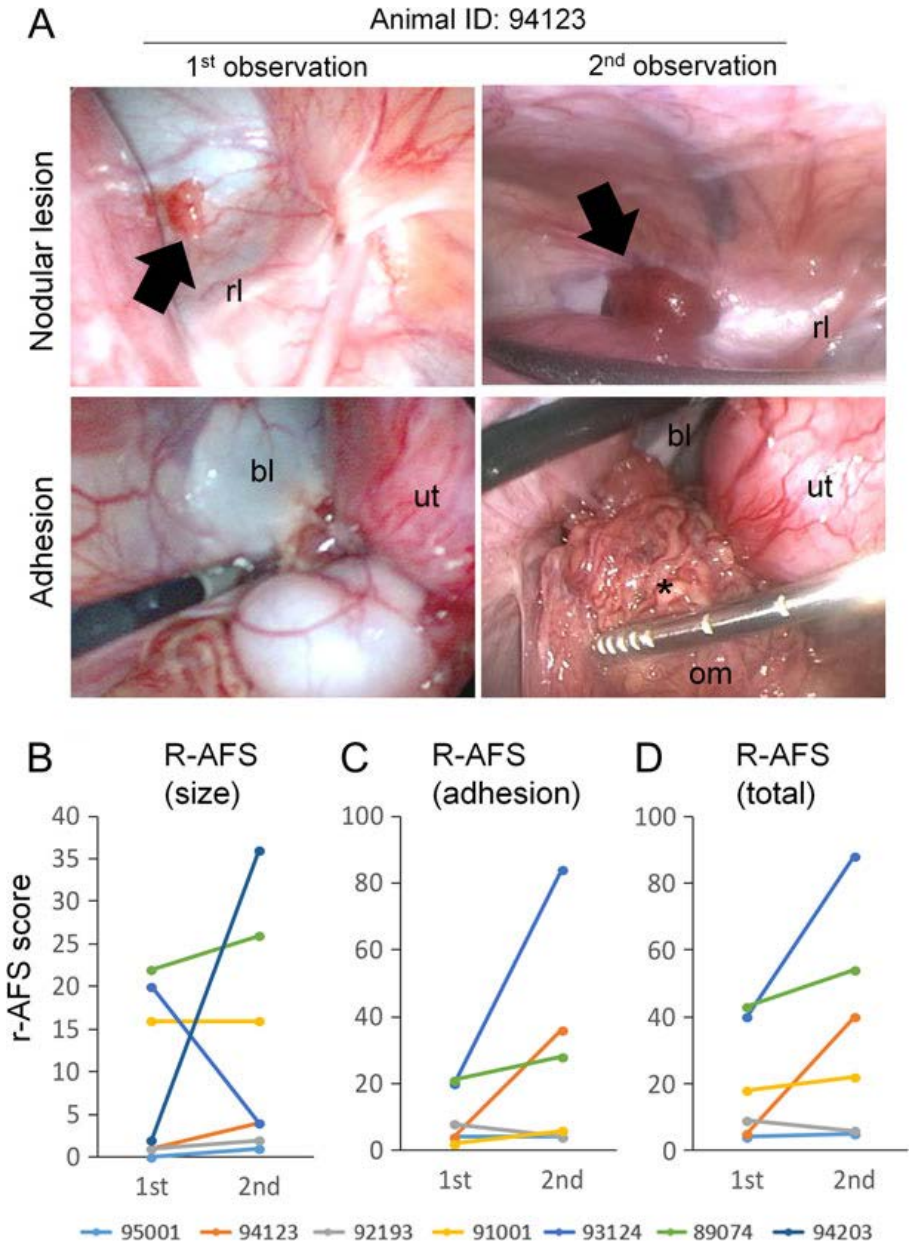


Figure 2-4. Time-course monitoring of endometriosis in cynomolgus monkeys by laparoscopy. (A) Representative images of laparoscopy during time-course monitoring, showing a nodular lesion (upper) and adhesion (lower). Lesion color changed from pink to red (arrow) and the adhesion progressed to the omentum (asterisk). (B–D) Disease monitoring using the revised American Fertility Society (r-AFS) score: size score (B), adhesion score (C) and total score (D). rl, round ligament.

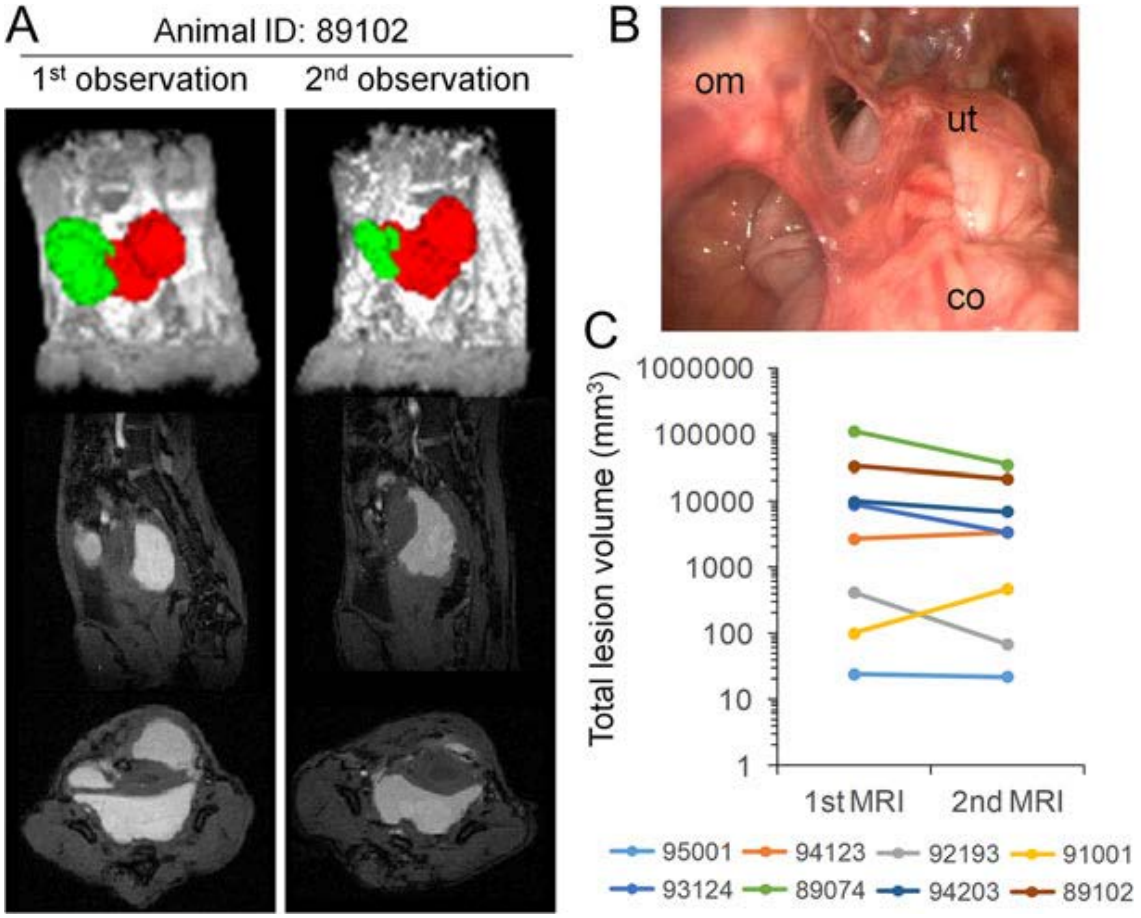


Figure 2-5. Time-course monitoring of endometriosis in cynomolgus monkeys by MRI. (A) Representative MRI result during time-course monitoring (animal 89102). The right ovarian cyst (in green) can be clearly distinguished from the cysts on the left and in the center (in red). (B) Laparoscopic image of the same animal as in A (89102), in which the extent of the cyst could not be observed because it was blocked by a severe adhesion. (C) Time courses of total lesion volume. co, colon.

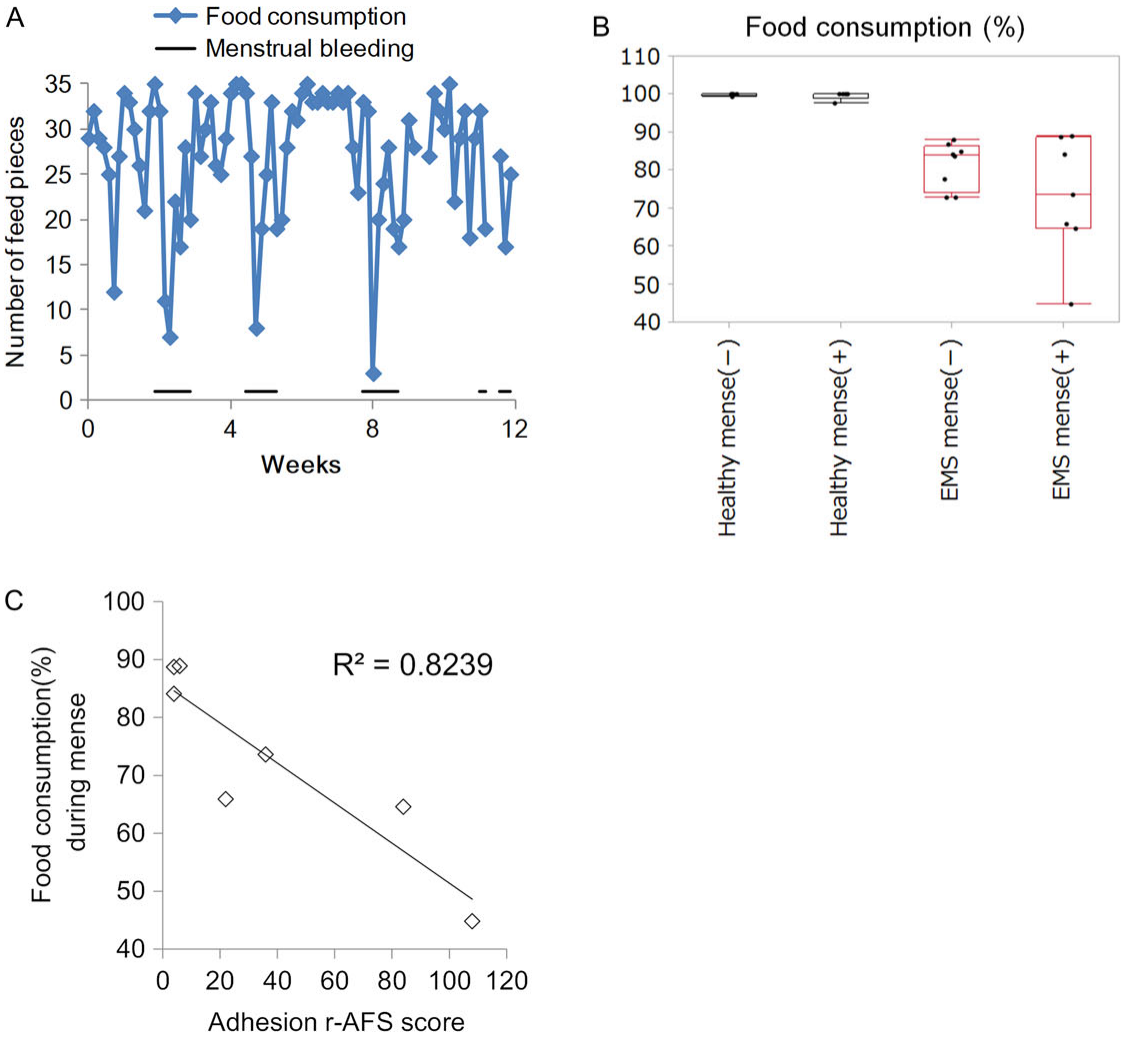
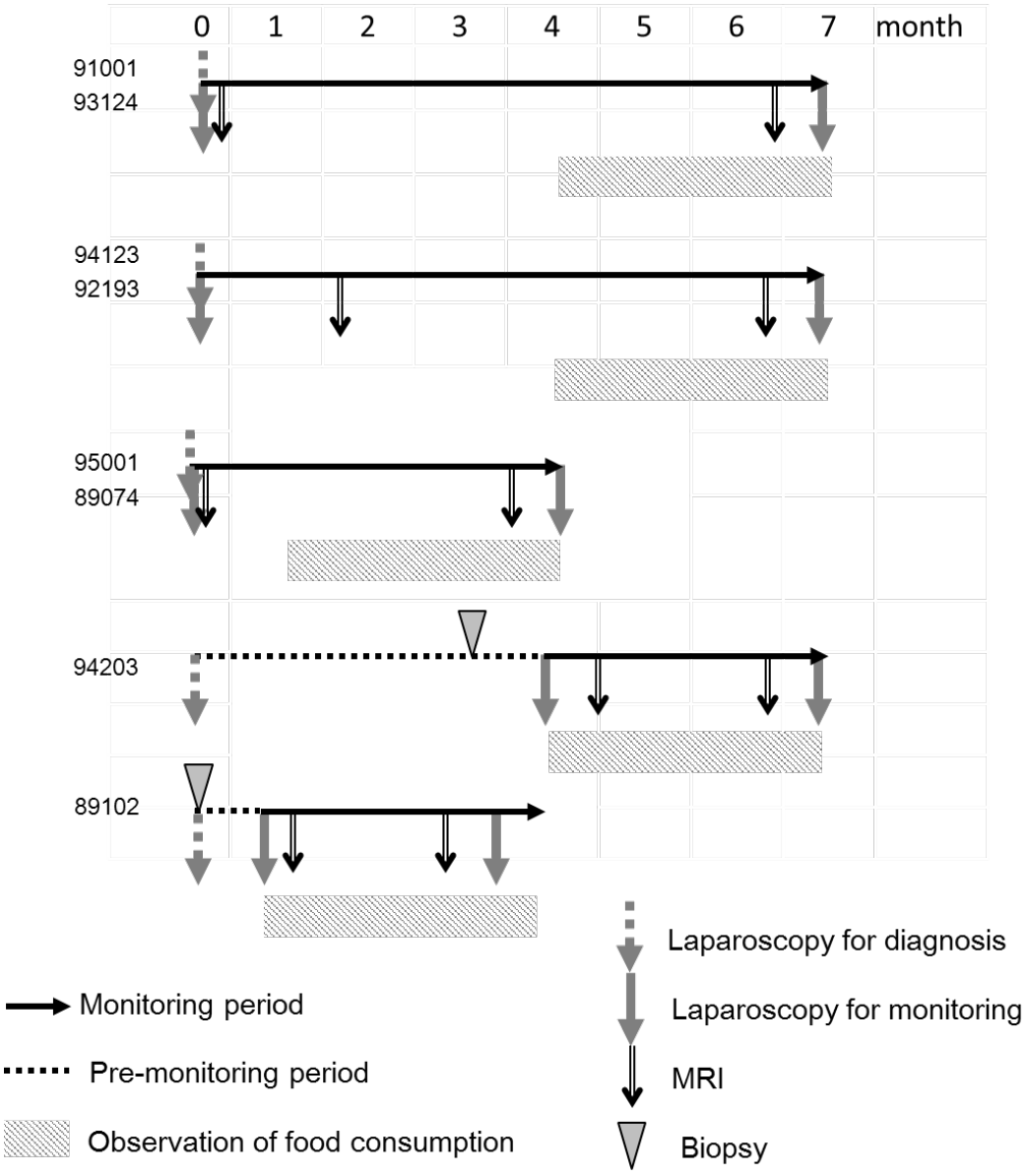


Figure 2-6. Food consumption and its relationship with the menstrual cycle and adhesion r-AFS score in cynomolgus monkeys. (A) Representative data of time-course changes in food consumption and menstrual bleeding (animal 93124). (B) Mean of food consumption (%) in periods with or without menses in healthy monkeys and monkeys with endometriosis. Bars in the box-and-whisker plots from the top: maximum, upper quartile, median, lower quartile, and minimum. Dot: mean of individual data for each animal. (C) Correlation of food consumption during menses (%) and adhesion r-AFS score (n = 7).



Supplemental Figure 2-1. Schedule for monitoring disease status in eight monkeys. Laparoscopic monitoring started from the diagnostic laparoscopy, except for biopsied monkeys (94203, 89102), which started from 3rd to 4th weeks after biopsy to avoid the effect of biopsy. The first MRI was taken after 1st monitoring laparoscopy and 2nd MRI was taken before 2nd monitoring laparoscopy. Food consumption was analyzed data taken over 12 weeks and records started between 9 and 12 weeks before the 2nd monitoring laparoscopy.

General discussion

This study showed that several aspects of the gross and histological characteristics of spontaneous endometriosis in cynomolgus monkeys, such as the three layers and infiltrating macrophages, were similar to those in humans. It is particularly noteworthy that several disease components in cynomolgus monkeys might contribute to endometriosis-related pain and recurrence of the disease.

Spontaneous endometriosis in cynomolgus monkeys satisfied the three requirements of an endometriosis animal model: disease screening was achieved using CA125, palpation, and fecal observation; disease status could be evaluated, and staging was applicable; and disease monitoring using laparoscopy and MRI was established. In addition, I found that food consumption might be a useful biological parameter related to the menstrual cycle and endometriosis-associated adhesions. Moreover, plasma cytokine levels were elevated, which was consistent with the observation of infiltrating immune cells, such as macrophages, in the endometriotic lesions.

Finally, I compared the characteristics of spontaneous endometriosis in baboons and cynomolgus monkeys as animal models for drug evaluation (Table 3-1). The main strength of the baboon model is that it is easy to observe because the body weight of female baboons ranges from 8 to 15 kg (D'Hooghe et al., 2009), whereas that of cynomolgus monkeys used in this study ranged from 2 to 5 kg. However, as a model for drug evaluation, the cynomolgus monkey model is superior since it is relatively easy to handle and analyze the general condition of cynomolgus monkeys; in addition, the mRNA and protein expression can be measured in cynomolgus monkeys. Moreover, the whole-genome sequencing of cynomolgus monkeys is available (Higashino et al., 2012), which

is useful for genetic characterization of this model. In addition, I reported, for the first time, that changes in food consumption in NHP might be linked to the menstrual cycle, which might be related to menstrual pain in clinical endometriosis. The relationship between the menstrual cycle and food consumption needs to be verified in baboons.

In conclusion, spontaneous endometriosis in cynomolgus monkeys could be a clinically relevant model that satisfies the requirements for evaluation of drugs for management of endometriosis with fibrosis and adhesions.

Table 3-1. Characteristics of endometriosis models in baboons and cynomolgus monkeys.

Items		Baboon	Cynomolgus monkey
Screening	Serum CA125	Not applicable (Serum CA125 was not elevated with endometriosis) (Falconer et al., 2005)	Applicable
	Palpation	May be applicable (There is a case report of diagnosis with palpation) (DaRif et al., 1984).	Applicable
	General condition	No report of monitoring general conditions (feces and food consumption etc) in the monkeys with endometriosis.	Applicable (laboratory setting is matured and individual monitoring of feces and food consumption is applicable)
		Menstruation was detectable over menstrual flow and perineal skin monitoring (D'Hooghe et al., 2009)	Menstruation was detectable with menstrual bleeding
Diagnosis with staging	Laparoscopic observation	Applicable (D'Hooghe et al., 1992)	Applicable
	Staging	Applicable with modified r-AFS score (D'Hooghe et al., 1996b)	Applicable with modified r-AFS score
Monitoring	Laparoscopic observation	Applicable (D'Hooghe et al., 1996b)	Applicable
	Lesion size and location by MRI	No report	Applicable
Pharmacological endpoint	Pain surrogate marker linked to menstrual cycles	No report	Food consumption
	Gene expression profiling	Limited to reported genes or need to be sequenced (whole genome sequence is not reported)	Commercial microarray is available and primers can be designed from sequence database (open access) (Higashino et al., 2012)
	Analysis of protein expression	Need to check the cross-reactivity	ELISA and Luminex kits are commercially available.

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References

American_Fertility_Society (1985). Revised American Fertility Society classification of endometriosis: 1985. Fertil Steril *43*, 351-352.

Ami, Y., Suzaki, Y., and Goto, N. (1993). Endometriosis in cynomolgus monkeys retired from breeding. J Vet Med Sci *55*, 7-11.

Asante, A., and Taylor, R.N. (2011). Endometriosis: the role of neuroangiogenesis. Annu Rev Physiol *73*, 163-182.

Barcena de Arellano, M.L., Arnold, J., Vercellino, G.F., Chiantera, V., Ebert, A.D., Schneider, A., and Mechsner, S. (2011a). Influence of nerve growth factor in endometriosis-associated symptoms. Reprod Sci *18*, 1202-1210.

Barcena de Arellano, M.L., Gericke, J., Reichelt, U., Okuducu, A.F., Ebert, A.D., Chiantera, V., Schneider, A., and Mechsner, S. (2011b). Immunohistochemical characterization of endometriosis-associated smooth muscle cells in human peritoneal endometriotic lesions. Hum Reprod *26*, 2721-2730.

Barcena de Arellano, M.L., and Mechsner, S. (2014). The peritoneum--an important factor for pathogenesis and pain generation in endometriosis. J Mol Med (Berl) *92*, 595-602.

Bazot, M., Darai, E., Hourani, R., Thomassin, I., Cortez, A., Uzan, S., and Buy, J.N. (2004). Deep pelvic endometriosis: MR imaging for diagnosis and prediction of extension of disease. Radiology *232*, 379-389.

Bergeron, C., Amant, F., and Ferenczy, A. (2006). Pathology and physiopathology of adenomyosis. Best Pract Res Clin Obstet Gynaecol *20*, 511-521.

Berkley, K.J., Rapkin, A.J., and Papka, R.E. (2005). The pains of endometriosis. Science *308*, 1587-1589.

Beste, M.T., Pfaffle-Doyle, N., Prentice, E.A., Morris, S.N., Lauffenburger, D.A., Isaacson, K.B., and Griffith, L.G. (2014). Molecular network analysis of endometriosis reveals a role for c-Jun-regulated macrophage activation. Sci Transl Med *6*, 222ra216.

Borrelli, G.M., Kaufmann, A.M., Abrao, M.S., and Mechsner, S. (2015). Addition of MCP-1 and MIP-3beta to the IL-8 appraisal in peritoneal fluid enhances the probability of identifying women with endometriosis. J Reprod Immunol *109*, 66-73.

Braundmeier, A.G., and Fazleabas, A.T. (2009). The non-human primate model of endometriosis: research and implications for fecundity. Mol Hum Reprod *15*, 577-586.

Burkle, B., Notscheid, N.K., Scheich, J., Hefler, L., Tempfer, C.B., Horvat, R., and Rezniczek, G.A. (2013). Spread of endometriosis to pelvic sentinel lymph nodes: gene expression analysis. Eur J Obstet Gynecol Reprod Biol *169*, 370-375.

Burney, R.O., and Giudice, L.C. (2012). Pathogenesis and pathophysiology of endometriosis. Fertil Steril *98*, 511-519.

Chapron, C., Bourret, A., Chopin, N., Dousset, B., Leconte, M., Amsellem-Ouazana, D., de Ziegler, D., and Borghese, B. (2010). Surgery for bladder endometriosis: long-term results and concomitant management of associated posterior deep lesions. Hum Reprod *25*, 884-889.

Cline, J.M., Wood, C.E., Vidal, J.D., Tarara, R.P., Buse, E., Weinbauer, G.F., de Rijk, E.P., and van Esch, E. (2008). Selected Background Findings and Interpretation of Common Lesions in the Female Reproductive System in Macaques. Toxicol Pathol *36*, 142s-163s.

Cornillie, F.J., D'Hooghe, T.M., Bambra, C.S., Lauweryns, J.M., Isahakia, M., and Koninckx, P.R. (1992). Morphological characteristics of spontaneous endometriosis in the baboon (Papio anubis and Papio cynocephalus). Gynecol Obstet Invest *34*, 225-228.

D'Hooghe, T.M., Bambra, C.S., Cornillie, F.J., Isahakia, M., and Koninckx, P.R. (1991). Prevalence and laparoscopic appearance of spontaneous endometriosis in the baboon (Papio anubis, Papio cynocephalus). Biol Reprod *45*, 411-416.

D'Hooghe, T.M., Bambra, C.S., De Jonge, I., Lauweryns, J.M., and Koninckx, P.R. (1996a). The prevalence of spontaneous endometriosis in the baboon (Papio anubis, Papio cynocephalus) increases with the duration of captivity. Acta Obstet Gynecol Scand *75*, 98-101.

D'Hooghe, T.M., Bambra, C.S., Isahakia, M., and Koninckx, P.R. (1992). Evolution of spontaneous endometriosis in the baboon (Papio anubis, Papio cynocephalus) over a 12-month period. Fertil Steril *58*, 409-412.

D'Hooghe, T.M., Bambra, C.S., Raeymaekers, B.M., and Koninckx, P.R. (1996b). Serial laparoscopies over 30 months show that endometriosis in captive baboons (Papio anubis, Papio cynocephalus) is a progressive disease. Fertil Steril *65*, 645-649.

D'Hooghe, T.M., Kyama, C.M., Chai, D., Fassbender, A., Vodolazkaia, A., Bokor, A., and Mwenda, J.M. (2009). Nonhuman primate models for translational research in endometriosis. Reprod Sci *16*, 152-161.

DaRif, C.A., Parker, R.F., and Schoeb, T.R. (1984). Endometriosis with bacterial peritonitis in a baboon. Lab Anim Sci *34*, 491-493.

De Graaff, A.A., Dirksen, C.D., Simoens, S., De Bie, B., Hummelshoj, L., D'Hooghe, T.M., and Dunselman, G.A. (2015). Quality of life outcomes in women with endometriosis are highly influenced by recruitment strategies. Hum Reprod *30*, 1331-1341.

Dehoux, J.P., Defrere, S., Squifflet, J., Donnez, O., Polet, R., Mestdagt, M., Foidart, J.M., Van Langendonckt, A., and Donnez, J. (2011). Is the baboon model appropriate for endometriosis studies? Fertil Steril *96*, 728-733 e723.

Dick, E.J., Jr., Hubbard, G.B., Martin, L.J., and Leland, M.M. (2003). Record review of baboons with histologically confirmed endometriosis in a large established colony. J Med Primatol *32*, 39-47.

Dunselman, G.A., Vermeulen, N., Becker, C., Calhaz-Jorge, C., D'Hooghe, T., De Bie, B., Heikinheimo, O., Horne, A.W., Kiesel, L., Nap, A.*, et al.* (2014). ESHRE guideline: management of women with endometriosis. Hum Reprod *29*, 400-412.

Falconer, H., Bambra, C.S., Chai, D., Cornillie, F.J., Hill, J.A., and D'Hooghe, T.M. (2005). The effect of endometriosis, cycle stage, lymphocyte suppression and pregnancy on CA-125 levels in peritoneal fluid and serum in baboons. Hum Reprod *20*, 3033-3038.

Fanton, J.W., and Hubbard, G.B. (1983). Spontaneous endometriosis in a cynomolgus monkey (Macaca fascicularis). Lab Anim Sci *33*, 597-599.

Fauconnier, A., Chapron, C., Dubuisson, J.B., Vieira, M., Dousset, B., and Breart, G. (2002). Relation between pain symptoms and the anatomic location of deep infiltrating endometriosis. Fertil Steril *78*, 719-726.

Fedele, L., Bianchi, S., Zanconato, G., Portuese, A., and Raffaelli, R. (2001). Use of a levonorgestrel-releasing intrauterine device in the treatment of rectovaginal endometriosis. Fertil Steril *75*, 485-488.

Giudice, L.C., and Kao, L.C. (2004). Endometriosis. Lancet *364*, 1789-1799.

Greene, R., Stratton, P., Cleary, S.D., Ballweg, M.L., and Sinaii, N. (2009). Diagnostic experience among 4,334 women reporting surgically diagnosed endometriosis. Fertil Steril *91*, 32-39.

Groothuis, P.G., and Guo, S.W. (2018). Drug Development in Endometriosis and Adenomyosis: It Takes More Than Just Good Science. Reprod Sci *25*, 1318-1329.

Harada, T., Iwabe, T., and Terakawa, N. (2001). Role of cytokines in endometriosis. Fertil Steril *76*, 1-10.

Higashino, A., Sakate, R., Kameoka, Y., Takahashi, I., Hirata, M., Tanuma, R., Masui, T., Yasutomi, Y., and Osada, N. (2012). Whole-genome sequencing and analysis of the Malaysian cynomolgus macaque (Macaca fascicularis) genome. Genome Biol *13*, R58.

Ito, T., Sakamaki, Y., Fujii, E., Misawa, Y., Suzuki, M., and Sugimoto, T. (2001). Endometriosis in a Rhesus Monkey. J Toxicol Pathol *14*, 313-313.

Khan, K.N., Kitajima, M., Hiraki, K., Fujishita, A., Sekine, I., Ishimaru, T., and Masuzaki, H. (2008). Immunopathogenesis of pelvic endometriosis: role of hepatocyte growth factor, macrophages and ovarian steroids. Am J Reprod Immunol *60*, 383-404.

Kishi, Y., Suginami, H., Kuramori, R., Yabuta, M., Suginami, R., and Taniguchi, F. (2012). Four subtypes of adenomyosis assessed by magnetic resonance imaging and their specification. Am J Obstet Gynecol *207*, 114 e111-117.

Koninckx, P.R., Ussia, A., Adamyan, L., Wattiez, A., and Donnez, J. (2012). Deep endometriosis: definition, diagnosis, and treatment. Fertil Steril *98*, 564-571.

Laux-Biehlmann, A., d'Hooghe, T., and Zollner, T.M. (2015). Menstruation pulls the trigger for inflammation and pain in endometriosis. Trends Pharmacol Sci *36*, 270-276.

Maroun, P., Cooper, M.J., Reid, G.D., and Keirse, M.J. (2009). Relevance of gastrointestinal symptoms in endometriosis. Aust N Z J Obstet Gynaecol *49*, 411-414.

McCluggage, W.G., Sumathi, V.P., and Maxwell, P. (2001). CD10 is a sensitive and diagnostically useful immunohistochemical marker of normal endometrial stroma and of endometrial stromal neoplasms. Histopathology *39*, 273-278.

Mechsner, S., Weichbrodt, M., Riedlinger, W.F., Kaufmann, A.M., Schneider, A., and Kohler, C. (2010). Immunohistochemical evaluation of endometriotic lesions and disseminated endometriosis-like cells in incidental lymph nodes of patients with endometriosis. Fertil Steril *94*, 457-463.

Merrill, J.A. (1968). Spontaneous endometriosis in the Kenya baboon (Papio doguera). Am J Obstet Gynecol *101*, 569-570.

Morotti, M., Vincent, K., Brawn, J., Zondervan, K.T., and Becker, C.M. (2014). Peripheral changes in endometriosis-associated pain. Hum Reprod Update *20*, 717-736.

Nishimoto-Kakiuchi, A., Netsu, S., Matsuo, S., Hayashi, S., Ito, T., Okabayashi, S., Yasmin, L., Yuzawa, K., Kondoh, O., Kato, A.*, et al.* (2016). Characteristics of histologically confirmed endometriosis in cynomolgus monkeys. Hum Reprod *31*, 2352-2359.

Nishimoto-Kakiuchi, A., Netsu, S., Okabayashi, S., Taniguchi, K., Tanimura, H., Kato, A., Suzuki, M., Sankai, T., and Konno, R. (2018). Spontaneous endometriosis in cynomolgus monkeys as a clinically relevant experimental model. Hum Reprod *33*, 1228-1236.

Odagiri, K., Konno, R., Fujiwara, H., Netsu, S., Ohwada, M., Shibahara, H., and Suzuki, M. (2007). Immunohistochemical study of osteopontin and L-selectin in a rat endometriosis model and in human endometriosis. Fertil Steril *88*, 1207-1211.

Odagiri, K., Konno, R., Fujiwara, H., Netsu, S., Yang, C., and Suzuki, M. (2009). Smooth muscle metaplasia and innervation in interstitium of endometriotic lesions related to pain. Fertil Steril *92*, 1525-1531.

Pirdel, L., and Pirdel, M. (2014). Role of iron overload-induced macrophage apoptosis in the pathogenesis of peritoneal endometriosis. Reproduction *147*, R199-207.

Sato, J., Doi, T., Kanno, T., Wako, Y., Tsuchitani, M., and Narama, I. (2012). Histopathology of incidental findings in cynomolgus monkeys (macaca fascicularis) used in toxicity studies. J Toxicol Pathol *25*, 63-101.

Siesto, G., Ieda, N., Rosati, R., and Vitobello, D. (2014). Robotic surgery for deep endometriosis: a paradigm shift. Int J Med Robot *10*, 140-146.

Story, L., and Kennedy, S. (2004). Animal studies in endometriosis: a review. ILAR J *45*, 132-138.

Takahashi, N., Yoshino, O., Maeda, E., Naganawa, S., Harada, M., Koga, K., Hiraike, O., Nakamura, M., Tabuchi, T., Hori, M.*, et al.* (2016). Usefulness of T2 star-weighted imaging in ovarian cysts and tumors. The journal of obstetrics and gynaecology research *42*, 1336-1342.

Tempfer, C.B., Wenzl, R., Horvat, R., Grimm, C., Polterauer, S., Buerkle, B., Reinthaller, A., and Huber, J.C. (2011). Lymphatic spread of endometriosis to pelvic sentinel lymph nodes: a prospective clinical study. Fertil Steril *96*, 692-696.

Vercellini, P., Vigano, P., Somigliana, E., and Fedele, L. (2014). Endometriosis: pathogenesis and treatment. Nat Rev Endocrinol *10*, 261-275.

Vigano, P., Candiani, M., Monno, A., Giacomini, E., Vercellini, P., and Somigliana, E. (2018). Time to redefine endometriosis including its pro-fibrotic nature. Hum Reprod *33*, 347-352.

Witmer, A.N., Dai, J., Weich, H.A., Vrensen, G.F., and Schlingemann, R.O. (2002). Expression of vascular endothelial growth factor receptors 1, 2, and 3 in quiescent endothelia. J Histochem Cytochem *50*, 767-777.

Yamanaka, A., Kimura, F., Takebayashi, A., Kita, N., Takahashi, K., and Murakami, T. (2012). Primate model research for endometriosis. Tohoku J Exp Med *226*, 95-99.

Zhang, Q., Duan, J., Liu, X., and Guo, S.W. (2016). Platelets drive smooth muscle metaplasia and fibrogenesis in endometriosis through epithelial-mesenchymal transition and fibroblast-to-myofibroblast transdifferentiation. Mol Cell Endocrinol *428*, 1-16.

List of Publications

1. **Ayako Nishimoto-Kakiuchi,** Sachiho Netsu, Sachi Okabayashi, Kenji Taniguchi, Hiromi Tanimura, Atsuhiko Kato, Masami Suzuki, Tadashi Sankai, Ryo Konno. Spontaneous endometriosis in cynomolgus monkeys as a clinically relevant experimental model. Human Reproduction, Vol.33, No.7 pp.1228-1236, 2018.

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