Editorial

Translational animal models for endometriosis research: a long and windy road

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Endometriosis is a chronic estrogen-dependent disease affecting 5–10% of women in reproductive age (1). Although it can be asymptomatic in a minority of the cases, the main symptoms and signs include chronic pelvic pain, dysmenorrhea, pain during intercourse, abnormal uterine bleeding and infertility, which may cause severe impact on patient's psychological wellbeing and quality of life (2). The disease is characterized by ectopic endometrial-like tissue (glands and stroma) that induces a chronic inflammatory response, adhesions, and scar tissue that alter pelvic anatomy (3). From the clinical point of view, endometriosis was subdivided in peritoneal superficial lesions, ovarian cysts (endometriomas) and deep infiltrating endometriosis, and usually scored according to the revised American Fertility Society classification (rAFS) (1) in four stages: minimal, mild, moderate and severe. Among the several theories proposed so far, retrograde menstruation was claimed as the potential reason why endometrial debris shed during menstrual cycle implants in the peritoneal cavity and proliferate; nevertheless, since retrograde menstruation was demonstrated in approximately 90% of healthy women, other pathogenic elements, including hormonal, immunological, genetic and epigenetic mechanisms may occur to allow the disease establishment and progression (4).

According to updated guidelines, the histological confirmation on surgical specimen is required for the diagnosis (1), which is sometimes significantly delayed when symptoms and signs are not adequately recognized by clinical examination and imaging. Current management relies on different hormonal drugs, including combined hormonal contraceptives, progestogens (cyproterone acetate, norethisterone acetate, danazol, medroxyprogesterone acetate oral or depot, dienogest), anti-progestogens such as gestrinone, levonorgestrel-releasing intrauterine system (LNG-IUS), gonadotropin releasing hormone analogues (nafarelin, leuprolide, buserelin, goserelin or triptorelin) with or without add-back therapies (combined hormonal contraceptives): nevertheless, to date there is no overwhelming and robust evidence to support one hormonal treatment over the others (1) and, in addition, hormonal drugs do not cure the disease, which usually recurs if administration is interrupted. When medical therapies are not enough to control symptoms and signs, surgical management by minimally invasive and fertility-sparing approach is mandatory to remove ectopic lesions, re-establish normal anatomy, reduce pelvic pain and improve reproductive outcomes (5).

Considering the lack of a definitive treatment and, most important, a limited knowledge of the disease's etiology, researchers tried to find animal models to add pieces of evidence to the understanding of pathogenic mechanisms and to find novel targets for therapy. In this regard,

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autologous or syngeneic rodent models, xenotransplantation of human endometrium into immunodeficient mice models and, to a lesser extent due to ethical reasons as well as high cost, nonhuman primate represent the most studied animal models for endometriosis. The most important difference among these models is that rats, mice, rabbits, and hamsters do not develop endometriosis spontaneously, whereas it occurs in nonhuman primate.

In the autologous or syngeneic rodent model, the immune system is intact, and this offers the possibility to study the cross-talk between the immune system and endometriotic cells within the peritoneal microenvironment, which was shown to play a key role in humans (6). In this model, uterine horns from female mice C57BL/6 (7), BALB/C (8) or Wistar rats (9) are removed by surgery. Usually endometrial fragments are collected by mechanical separation or dermal biopsy punch, afterwards they are suspended and injected in the peritoneal cavity or sutured to the peritoneal wall of the same animal. The usual size of endometrial fragments in mice range from 2 to 4 mm^2, whereas 5 mm^2 is considered standard in rats. As summarized elsewhere (10), the syngeneic rodent models were able to provide robust pieces of evidence especially regarding the efficacy of antiangiogenic compounds on microvessel diameter and density, volumetric blood flow to the lesion, and endometriotic lesion growth and survival. Interestingly, rats affected by induced endometriosis showed impaired fecundity, decreased litter size and embryos weight (11), suggesting a detrimental role on fertility similar to what is widely known in humans. In addition, peritoneal fluid of women with endometriosis, which is known to orchestrate a detrimental effect on fertility, was evaluated injecting it into the peritoneal cavity of laboratory animals: interestingly, this led to significantly decreased implantation rates in hamsters and in mice (12). Peritoneal fluid of women with endometriosis has been shown able also to alter cleavage of two-cell mouse embryos *in vitro* and murine sperm—oocyte interaction (12).

Another interesting approach was to remove endometrium from transgenic mice, steady expressing luciferase, and induce endometriosis in immunodeficient non-obese diabetic-severe combined immunodeficient (NOD-SCID) mice (7): in this way, the emission of light after luciferin injection directly correlates with lesion size. Finally, others used a model based on the dorsal skinfold of the hamster (13): first, the dorsal skinfold is lodged between two titanium frames; subsequently, a skin layer is detached, in order to expose striated muscle, and then covered with a coverslip; finally, the autologous transfer of endometrium on the striated muscle is used to induce endometriosis.

Beside syngeneic rodent models, xenotransplantation of human endometrium into immunodeficient mice model offers another possibility to investigate the disease: in previous reports, immunodeficient athymic nude lacking T-lymphocytes (14), SCID lacking B- and T-lymphocytes (15) and NOD-SCID (8) mice have been used. Once human endometrial fragments are obtained, the methods to induce the disease in the animal model are the same. On the one hand, this approach has the great advantage to investigate human endometrial responses to potential therapies *in vivo*; nevertheless, on the other hand, the lack of immunocompetence represents a significant factor that may limit the investigation of several molecular and cellular pathways. Just for example, athymic nude mice are characterized by high natural killer (NK) cell levels, which restrain human endometriotic lesion growth: according to previous data (16), in SCID mice or athymic nude mice with NK cell suppression, endometriotic lesions after induction were found in 100% of the samples and continued to proliferate even after 10 weeks from the first observation, whereas endometriotic foci were found in only 40% of the sample in case of athymic nude mice without NK suppression. In addition, mice are often given exogenous estradiol to support the growth of the human endometrial tissue: this is known to have also systemic effects on the animal, and should be taken into account for a proper data interpretation about therapeutic efficacy. In light of these findings, and taking into account the pivotal role of immune system at the maternal-fetal interface for correct embryo implantation and subsequent development of healthy pregnancy, xenotransplantation of human endometrium into immunodeficient mice has not been used so far for fertility studies.

Considering all these elements and potential limitations, researches addressed their attention to nonhuman primates such as rhesus monkeys and baboons (Papio anubis), because they menstruate in a cyclic pattern and develop endometriosis spontaneously, although it occurs with a significantly lower frequency than in humans. Interestingly, even induced endometriosis in nonhuman primates leads to a reduction in fertility, similar to what occurs in humans: indeed, decreased fertilization and term pregnancy rates have been shown after surgical induction of endometriosis in cynomolgus monkeys (Macaca fascicularis) with moderate or severe disease (17). In addition, subfertility associated to endometriosis was correlated with stage disease also in

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baboons (18).

Accumulating evidence suggests that poor reproductive outcomes after experimentally induced and spontaneous endometriosis are due to a non-mutually exclusive combination of altered follicle maturation, ovulation, fertilization, implantation, or embryonic development (12). In particular, in monkeys affected by induced endometriosis, when adnexal adhesions were on the same ovary where ovulation occurred, no term pregnancies were noted; in contrast, when there were no adnexal adhesions, term pregnancy rates in monkeys with endometriosis and in control monkeys with adipose tissue implants were overlapping (17). In addition, animals with moderate and severe disease showed a high rate of luteinized unruptured follicle syndrome, which has been suggested as the main cause of endometriosis-associated infertility in monkeys (17). Apart from altered pelvic anatomy and ovulatory function in both induced and spontaneous endometriosis in nonhuman primates, impaired endometrial function may also play a key role for observed subfertility. It was already demonstrated that *HOXA10*, a gene essential to address the correct implantation, significantly increases its expression during the luteal phase in women; nevertheless, women with endometriosis did not show progesterone-induced upregulation of *HOXA10* gene during implantation window (19). Similarly, in the baboon *HOXA10* protein has been found significantly reduced in eutopic endometrium 12 and 16 months after induction of the endometriosis (20).

In this regard, it is crystal clear that nonhuman primate may represent the best animal models in endometriosis research, taking into account their similarity to humans regarding phylogenetics, reproductive physiology and anatomy, and the presence of spontaneous endometriosis histologically and macroscopically identical to its human counterpart. Although spontaneous endometriosis could be considered the best possible model, moderate-severe disease associated with cystic ovarian lesions is not commonly found in nonhuman primates (21). Furthermore, only in some species (Papio anubis and Macaca fascicularis) the menstrual cycles are approximately every 4 weeks and spontaneous endometriosis is considered relevant. Indeed, diagnosis of the spontaneous disease in nonhuman primate models is difficult, since (as in humans) there is a lack of reliable noninvasive methods for the early detection, and a large number of animals is required (22).

In this scenario, the recent article by Nishimoto-Kakiuchi *et al.* (23) provides novel and important insights from a nonhuman primate for translational research in endometriosis: in this work, they systematically evaluated screening, diagnosis, staging, and monitoring in a population of cynomolgus monkeys. The proposed protocol is methodologically robust and has the advantage to use an animal model with smaller body size than baboons, making easier the control and handling in laboratory setting. Nevertheless, the same element may represent also a limit, considering that small body size may not allow (or make it difficult to perform) repetitive blood sampling, repetitive laparoscopies, complex experimental surgeries, and vaginal trans cervical access to the uterine cavity compared to Papio species. Compared to other species, parameters such as mRNA and protein expression and the fully sequenced genome are available in cynomolgus monkeys (24), and these might represent efficient methods to further clarify endometriosis pathogenesis and develop novel drugs.

As fully detailed by Nishimoto-Kakiuchi *et al.* (23), screening was performed with a primary selection of the general population based on regular menstruation (5 times per year or more) and with a secondarily selection based on high values of serum Ca-125 (>35 IU/L) and/or aberrance found on palpation during routine medical examination. Diagnostic accuracy was based on histological confirmation of endometriosis or intraoperative findings. Due to the overall positive predictive value (PPV) of 51.7%, authors investigated other biological parameters and found that the observation of feces was a good item for screening. Furthermore, they appropriately suggested that animals in which the first diagnostic examination fails to detect endometriosis should be kept under monitoring, because of the high probability of endometriosis development later in time. Finally, for the first time Ca-125 levels were found correlated with the presence of endometriomas in monkeys, while it was not confirmed in baboons (25) and monkeys with only peritoneal endometriosis. Considered altogether, Nishimoto-Kakiuchi *et al.* (23) found an overall sensitivity and PPV of 66.7% and 76.9% for medical examination and observation of feces alone or in combination, respectively. These parameters are obtained as part of usual laboratory practice, therefore are available without further evaluation, and represent a promising screening methodology. Nevertheless, this screening allowed the diagnosis of 15 subjects with spontaneous endometriosis among 614 female cynomolgus monkeys of reproductive age, with a detection rate lower compared to a prevalence of endometriosis after autopsy of 28.7%.

Diagnosis and staging were performed with surgery (despite the small body size of this model), both by

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laparoscopy and open surgery, with tissue sampling performed in almost half of the subjects and evaluating even small lesions. Staging was performed calculating the r-AFS score, assigning disease stage for each animal, although the scoring system was optimized for monkeys: the criteria for lesion size was changed (to 5 and 15 mm) because cynomolgus monkeys are smaller than humans; the item of adhesion at the vesicouterine pouch was added (complete, partial, open), similar to that for the pouch of Douglas, because adhesions at this pouch were observed more frequently in cynomolgus monkeys than in humans.

Monitoring was performed with both surgery and magnetic resonance imaging (MRI). The two methods reported results consistent with each other. Interestingly, MRI has been found better than laparoscopy in monitoring cystic lesion. Nevertheless, due to the limited number of subjects that underwent laparoscopy and MRI, further data are needed to confirm these results. Similarly, modified r-AFS resulted effective, although used only in 9 subjects, and further studies are needed. Food consumption monitoring during the menstrual phase, that represents a plausible "biological response" to pain in cynomolgus monkeys, could be one of the parameters to improve detection of spontaneous endometriosis. Interestingly, Nishimoto-Kakiuchi *et al.* (23) confirmed not only that food consumption during menstruation was decreased in case of endometriosis, but also that this element correlates with r-AFS stage modified for monkeys.

In summary, spontaneous endometriosis in cynomolgus monkeys described in this study could be considered a useful animal model to study etiology, development, progression of the disease and potentially to assess drug efficacy. However, further investigation to attest effectiveness of "biological response", correlating with endometriosis and its symptoms, may allow to improve diagnostic accuracy in nonhuman primate models and early detection, aligning with the primary end points of clinical endometriosis research in humans.

In conclusion, although cynomolgus monkey is a promising model, we take the opportunity to underline that nonhuman primate model choice should be always based taking into account study design and hypothesis tested, with the aim to optimize resources and meet the highest possible standards with respect to animal welfare.

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Footnote

Conflicts of Interest: The authors have no conflicts of interest to declare.

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