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Long-term follow-up of the effect of oral dienogest and dienogest/ethinylestradiol treatment on cell-free DNA levels in patients with deep endometriosis

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Abstract

Background Endometriosis is currently considered a systemic inflammatory disease and different non-invasive inflammatory markers, such as cell-free DNA (cfDNA), have recently been evaluated. Hormonal treatments are frequently prescribed as first-line treatments to improve symptoms, reduce lesions and improve the quality of life of patients with endometriosis. The most frequently used hormonal treatments are estroprogestins and progestins due to their effectiveness and well-tolerated clinical profile. However, the impact these hormonal treatments may have on these markers has yet to be determined. The aim of this study was to assess whether cfDNA levels are modified under the two main first-line hormonal treatments in patients with deep endometriosis (DE).

Methods Ninety patients diagnosed with DE were analyzed in this prospective, observational study. Forty-five received daily oral treatment with dienogest 2 mg, and 45 with 2 mg dienogest/30 µg ethinylestradiol. Plasma cfDNA levels were evaluated by fluorescent assay prior to initiation of treatment and at 6 and 12 months of treatment.

Results An increase in cfDNA levels was observed during the follow-up at 6 and 12 months. However, these higher levels were only statistically significant at 12 months of treatment. The increase of cfDNA levels was similar with both treatments.

Conclusion Higher cfDNA levels were observed in DE patients at 12 months of oral hormonal treatment showing similar results with dienogest or dienogest/ethinylestradiol. This increase could be explained by apoptosis of the endometriosis foci due to the treatment.

Background

Endometriosis is a chronic hormone-dependent disorder caused by the presence of extrauterine endometrial-like tissue [1], with a prevalence of 10% in women of reproductive age and co-existing in up to 30% of cases with adenomyosis and in up to 50% of cases with fertility disorders [2]. Among the different types of endometriosis, deep endometriosis (DE) is considered the most severe form [1, 2]. In recent years, evidence has led to the belief that endometriosis may be a systemic inflammatory

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disorder rather than a condition limited to the pelvis as was once thought [3]. This shift in paradigm has promoted the study of new markers for this disease [4]. In the last decade, there has been growing interest in the search for biomarkers which could lead to a minimally invasive diagnosis or have prognostic value. This has opened a new field of study, known as the omic sciences, which focuses on the study of molecules that may play a role in the etiopathogenesis of the disease or serve as diagnostic or prognostic factors. In the case of endometriosis, the biomarkers studied range from glycoproteins, growth factors, microRNAs, cytokines as well as proteins related to angiogenesis and the immune system [5–7].

Cell-free DNA (cfDNA) is characterized by non-cell-bound, double-stranded DNA fragments present in human plasma or serum [8]. The discovery of cfDNA in plasma brought about many innovations in several areas of medicine, becoming a marker of growing interest due to the possible clinical applications and expanding the possibilities of non-invasive diagnosis and prognosis [9]. Increased cfDNA concentrations have been found in inflammatory conditions, such as systemic lupus erythematosus and rheumatoid arthritis [10, 11], trauma [12] and some cancers [9, 13]. Furthermore, cfDNA from circulating cells is considered a potential biomarker of endometriosis [14]. Recently, elevated cfDNA levels were detected in patients diagnosed with endometriosis compared with healthy individuals [14] but other studies did not find such differences [15, 16].

How cfDNA is released into the bloodstream is still poorly understood. Studies show that cellular events, such as necrosis, apoptosis and secretion by the cells themselves, determine the number of cfDNA fragments, which can be released through active or passive mechanisms. cfDNA might act as a potential signaling molecule under specific conditions [17]. A well-known source of cfDNA is neutrophil extracellular traps (NETs), which represent an ancient and important part of our innate immune defense system [18]. NETs are composed of remodeled extracellular DNA fibers that are released by neutrophils in response to pathogenic triggers [19] and have been found to be increased in patients with endometriosis [20, 21].

Hormonal treatments are currently considered the first-line treatment for endometriosis because they improve symptoms, reduce lesions and improve the quality of life of more than two thirds of the patients [22]. The most frequently used hormonal treatments are estroprogestins and progestins due to their effectiveness and well-tolerated clinical profile and they are usually prescribed based on a shared patient–physician decision-making approach [22]. However, there are few studies and very little knowledge about the mechanisms of action of first-line hormonal treatments in patients with endometriosis. Furthermore, it remains to be determined if there is any biomarker of response to hormonal treatment. Therefore, the objective of this prospective observational study was to perform a long-term follow-up to evaluate the modifications induced by oral estroprogestin versus progestin treatment on cell-free DNA levels in patients with DE, the most severe type of endometriosis.

Materials and methods

Study design

A longitudinal, prospective, observational, single-center study was conducted at the Department of Gynecology of the Hospital Clinic of Barcelona, a tertiary university hospital in Spain and a referral center for the diagnosis and treatment of endometriosis. Patient recruitment was performed between October 2021 and November 2022. The study was approved by the local Ethical Committee (HCB/2020/1445 19 March 2021), according to prevailing regulations. Written informed consent was obtained from all participants.

The endpoint of this study was to investigate the impact of the most frequently used hormonal treatment for DE on cfDNA levels. Oral daily continuous progestin (dienogest 2 mg) and oral daily continuous estroprogestin (2 mg dienogest/30 µg ethinylestradiol) were evaluated. A shared decision-making approach used in routine medical practice, that considers the individual preferences, side effects, individual efficacy, costs, and availability, was carried out when counseling the patients on the choice between the two types of hormonal treatments. Patients were consecutively allocated to the dienogest group or the dienogest/ethinylestradiol group according to the shared decision-making process. If the patient refused to participate in the follow-up or blood sample obtention or was lost to follow-up, the next candidate to start oral treatment was allocated. Patient recruitment stopped when a minimum of 45 patients per treatment group and the 12-month follow-up was achieved. The estroprogestin treatment was composed of one active tablet of 2 mg dienogest/30 µg ethinylestradiol daily in a flexible extended regimen with cycles of 120 consecutive days of active tablets followed by a 4-day tablet-free interval, either after 120 days or after 3 consecutive days of spotting [23, 24]. The progestin treatment consisted of 2 mg/24 h of dienogest prescribed in a continuous manner [25]. Both treatments, types and doses, were chosen because they are the most frequently prescribed in daily practice to endometriosis patients due to their efficacy, tolerability and universal healthcare funding.

Participants

Participants needed to be aged between 18 and 45 years and have a diagnosis of at least one DE foci in the anterior or posterior compartment of the pelvis confirmed through a specialized transvaginal ultrasound (TVS) or magnetic resonance imaging. Eligible individuals were not candidates for surgical intervention and had not undergone hormonal treatment in the 6 months preceding the study. Exclusion criteria included a history of current or past malignancies, endocrine disorders, cardiovascular diseases, and other systemic illnesses, as well as pregnancy or breastfeeding within 6 months prior to sample collection. Additional exclusion factors were a body mass index (BMI) exceeding 30 kg/m^2, premature ovarian insufficiency or menopausal status, endometrial hyperplasia or polyps, uterine leiomyomas, and any inflammatory or infectious conditions occurring within 6 months before sample collection.

Study procedures

Venous blood samples were collected in tubes containing 3.8% trisodium citrate (1/9 vol/vol; BD Biosciences) at recruitment and at 6 and 12 months of follow-up by an appropriate venipuncture technique to minimize hemolysis during blood collection. Platelet-free plasma was immediately obtained by double centrifugation, first at 2000×*g* for 10 min at 22 °C and then at 5000×*g* for 10 min at 4 °C. Samples were visually inspected to rule out the presence of hemolysis. Plasma was aliquoted, snap-frozen in a mixture of dry ice/ ethanol (1/2 vol/vol) and stored at − 80 °C until use.

Circulating double-stranded DNA (dsDNA/cell-free DNA) was determined.

Plasma dsDNA/cell-free DNA was quantified in duplicate by a fluorescent assay using the Quant-iT™ PicoGreen dsDNA reagent. The tests were performed according to the manufacturer's instructions: first, to quantify the concentrations of double-stranded DNA (dsDNA), a five-point standard curve was established using dilutions ranging from 1 to 1000 ng/ml of the lambda DNA standard (100 µg/ml) provided. Then, 100 µl of blank (TE buffer), standard DNA dilutions or plasma samples were pipetted into a white 96-well plate. Thereafter, 100 µl of the PicoGreen working solution (PicoGreen reagent diluted 1:200 in TE buffer) was pipetted into each well, mixed well and incubated at room temperature for 2 to 5 min protected from light. Samples were tested in duplicate.

The absorbance of the samples was measured at an excitation wavelength of 480 nm and an emission wavelength of 520 nm utilizing a fluorescence spectrophotometer (Fluoroskan Ascent). The fluorescence intensity was determined by subtracting the fluorescence reading of the reagent blank from the readings of each sample. The results were reported in nanograms per milliliter (ng/ml) of DNA.

All patients underwent high-resolution 2D-3D TVS using an endovaginal probe (type RIC5-9, Voluson S10, GE Healthcare, Milwaukee, WI, USA). The diagnosis of DE and adenomyosis (AD) were established following the International Deep Endometriosis Analysis (IDEA) group consensus for DE [23] and the Morphological Uterus Sonographic Assessment (MUSA) group consensus for AD [27] as described previously [28, 29]. The IDEA TVS examination protocol consists of four steps: (1) evaluation of the uterus and adnexa; (2) evaluation of TVS soft markers; (3) assessment of the status of the pouch of Douglas using the real-time ultrasound-based sliding sign; and (4) assessment of DE nodules in the anterior and posterior compartments. To assess the anterior compartment, the transducer is positioned in the anterior fornix of the vagina. If bladder endometriosis is suspected on the basis of symptoms, patients should be asked not to empty their bladder completely before the ultrasound examination. A slightly filled bladder facilitates evaluation of the walls of the bladder and detection and description of endometriotic nodules. Finally, the transducer is positioned in the posterior fornix of the vagina and slowly withdrawn through the vagina to allow visualization of the posterior compartment. We recommend the use of a rectal enema the day before and the same day of the ultrasound examination to eliminate fecal residue and gas in the rectosigmoid.

Dysmenorrhea, non-menstrual pelvic pain, dyspareunia, dyschezia and dysuria were assessed at baseline and at the 6- and 12-month follow-ups using a numerical rating scale (NRS) from 0 to 10, where 0 means "no pain" and 10 "the worst pain".

Statistical analysis

As this was a preliminary study to investigate the impact of first-line oral hormonal treatment on cfDNA levels in endometriosis patients, the sample size was decided arbitrarily, albeit in keeping with previous studies analyzing cfDNA in endometriosis. A minimum of 45 patients per treatment group was proposed [14–16]. Categorical variables were expressed as count and percentages, and continuous variables as mean and standard deviation. The distribution of categorical variables was compared with the Chi-square test, and quantitative variables with the ANOVA test using the post hoc Bonferroni multiple comparison test and Student *t*-test, when appropriate. Statistical significance was set at $p < 0.05$. Statistical analysis was performed with the Statistical Package for the

Social Sciences software, release 27.0 for Windows (SPSS, Chicago, IL, USA).

Results

A total of 108 patients diagnosed with DE who did not meet surgical criteria were recruited for participation in the study. Figure 1 illustrates the patient inclusion and drop-out throughout the study.

Finally, a total of 90 patients were recruited and included in the final analysis; 45 received oral treatment with dienogest and 45 received dienogest/ethinylestradiol. cfDNA levels were determined at baseline and at 6 and 12 months of follow-up. A total of eight patients were excluded due to loss to or incomplete follow-up. Among these patients, seven abandoned the treatment before the first follow-up at 6 months due to non-severe side effects, such as persistent spotting ($n=5$) and/or weight gain ($n=3$), and/or headaches ($n=3$) and were excluded from the analysis. One patient was lost to follow-up before the 6-month follow-up and was excluded.

Table 1 Demographic and clinical data of the two study groups

	Dienogest/ethinylestradiol group ($n=45$)	Dienogest group ($n=45$)	*p* value
Age (years)	34.1 ± 6.4	36.4 ± 5.1	0.1
Nulliparous	31 (46.3)	29 (48.9)	0.2
BMI (kg/m^2)	22.6 ± 2.1	23.1 ± 1.9	0.1
History of infertility	10 (22.2)	12 (26.7)	0.1
Adenomyosis	14 (31.3)	15 (33.3)	0.9
Ovarian endometrioma	23 (51.1)	19 (42.2)	0.7
Rectosigmoid DE	15 (33.3)	17 (37.7)	0.9
Torus DE	25 (55.5)	22 (48.9)	0.3
Uterosacral ligament DE	33 (73.3)	34 (75.5)	0.6

Estroprogestin Group: women diagnosed with deependometriosis receiving oral hormonal treatment with a flexible extended regimen of ethinylestradiol 0.03 µg + dienogest 2 mg/day; Progestin Group: were diagnosed with deep endometriosis and receiving oral dienogest 2 mg/day. Results expressed as number and percentage or mean ± standard deviation. *BMI* body mass index, *DE* deep endometriosis

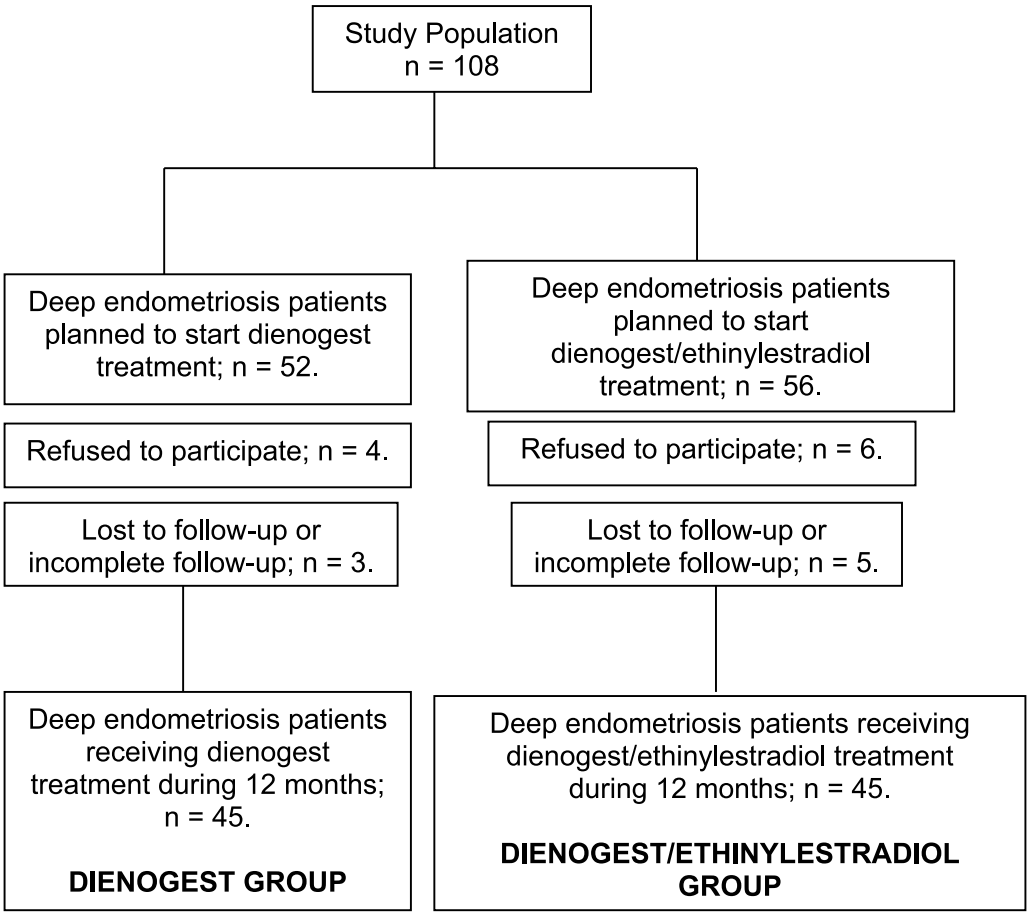

Fig. 1 Flowchart of patient inclusion and drop-out

No differences were found between groups in terms of mean age, BMI, nulliparity, sterility or concomitant AD (Table 1).

The types of endometriosis lesions were similar in both groups (Table 1). The NRS of dysmenorrhea, non-cyclic pelvic pain, dyspareunia, dyschezia and dysuria decreased over time and was similar in both groups at baseline and at the 6- and 12-month follow-ups (Table 2). There were no reports of severe side effects.

The two groups showed no differences in baseline cfDNA levels (ng/ml) (dienogest group: 143.6 ± 44.2; dienogest/ethinylestradiol group: 142.1 ± 41.7). With respect to the impact of hormonal treatment on cfDNA levels, an increase was observed at the 6-month follow-up (dienogest group: 147.9 ± 44.1; dienogest/ethinylestradiol group: 149.1 ± 33.6) and at 12 months (dienogest group: 175.3 ± 41.5; dienogest/ethinylestradiol group: 166.7 ± 45.3) in both groups (Fig. 2 and Table 3).

This increase was statistically significant when comparing the baseline status with the results at 12 months of undergoing hormonal treatment (Fig. 2 and Table 3). However, there were no differences between cfDNA levels at baseline and at the 6-month follow-up (Fig. 2 and Table 3). The difference was also not statistically significant when comparing the group receiving estroprogestin versus progestin treatment (Fig. 2).

The subanalysis of cfDNA levels among patients with and without concomitant AD or history of sterility showed no statistically significant differences (data not shown).

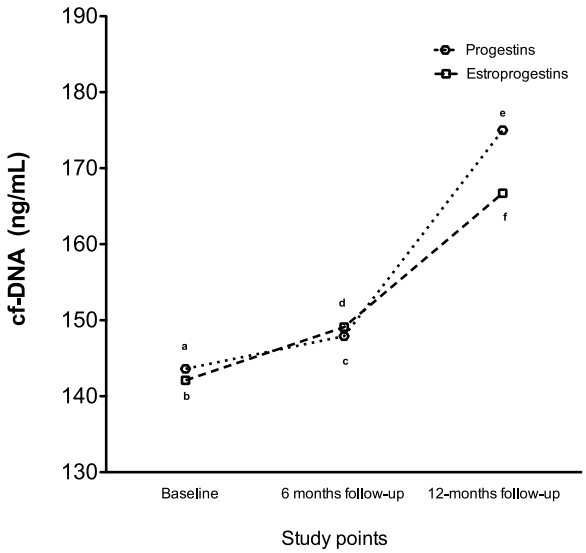

Fig. 2 Cell-free DNA levels (ng/ml) at baseline, 6-month and 12-month of follow-up in deep endometriosis patients receiving an oral flexible extended regimen of dienogest/ethinylestradiol versus dienogest. cf-DNA: cell free DNA; dienogest/ethinylestradiol Group: women diagnosed with deep endometriosis receiving oral hormonal treatment with a flexible extended regimen of ethinylestradiol 30 ug/dienogest 2 mg/day; Dienogest Group: were diagnosed with deep endometriosis and receiving oral Dienogest 2 mg/day. Results expressed as mean ± standard deviation. NS: not statistically significant. Superscripts show statistical differences: $p = NS^{a-b}$, $p = NS^{d-c}$, $p = NS^{a-b,c-d}$, $p = NS^{d-c,e-f}$, $p < 0.01^{a-b,f}$, $p < 0.04^{a-b,e}$

Discussion

This preliminary study is the first to compare molecular changes induced by the two main oral first-line hormonal treatments, progestins versus estroprogestins, in an extended regimen with a long-term follow-up. cfDNA plasma levels increased during follow-up in patients with DE receiving oral estroprogestins or progestins, being significant at 12 months of treatment. cfDNA levels did not differ between patients with or without AD or with a history of infertility.

There is controversy in the literature regarding cfDNA levels in endometriosis and their value as a biomarker of endometriosis. Several studies have reported increased cfDNA and NETs in endometriosis patients [14, 20, 21], whereas others have not or have only found increases in a specific phenotype of the disease [15, 16].

Hormonal treatments are currently used worldwide as an effective first-line treatment for patients

Table 2 Effect of hormonal treatments on symptomatic evaluation at baseline, and at 6 and 12 months of follow-up in patients with deep endometriosis

Hormonal treatment	Variables	Baseline	6-month follow-up	12-month follow-up	*p*-value
Ethinylestradiol 0.03 ug + Dienogest 2 mgr/day*	Dysmenorrhea**	9.1 ± 1.6^{a}	2.7 ± 2.1^{b}	0.2 ± 0.8^{c}	$^{a-b, b-c, a-c}$ $p < 0.001$
	Non-menstrual pelvic pain**	6.8 ± 1.9^{a}	1.7 ± 1.6^{b}	0.4 ± 1.1^{c}	$^{a-b, b-c, a-c}$ $p < 0.001$
Dienogest 2 mgr/day*	Dysmenorrhea**	8.9 ± 1.7^{a}	1.9 ± 2.1^{b}	0.1 ± 0.6^{c}	$^{a-b, b-c, a-c}$ $p < 0.001$
	Non-menstrual pelvic pain**	6.7 ± 1.4^{a}	1.5 ± 1.2^{b}	0.6 ± 0.9^{c}	$^{a-b, b-c, a-c}$ $p < 0.001$

Results expressed as number and percentage or mean ± standard deviation

Variables of pain are expressed as mean Numerical Rating Scale from 0 to 10

* The comparison of variables in all study points between both hormonal treatments showed no statistically significant differences

** Numerical rating scale from 0 to 10, where 0 means "no pain" and 10 "the worst pain"

Table 3 Means and 95% confidence intervals (CI) of cell-free DNA levels (ng/mL) at baseline, and at 6- and 12-month follow-up in deep endometriosis patients receiving an oral flexible extended regimen of dienogest/ethinylestradiol versus dienogest

Hormonal treatment	Baseline	6-month follow-up	12-month follow-up	*p*
Ethinylestradiol 30 µg + Dienogest 2 mgr/day*	142.1 (58.6–225.5)[a]	149.1 (75.9–222.3)[b]	166.7 (75.9–257.5)[c]	$p<0.01^{a-c}$, $p=NS^{a-b,b-c}$
Dienogest 2 mgr/day*	143.6 (55.2–232)[d]	147.9 (35.7–260.1)[e]	175.0 (93–258)[f]	$p<0.04^{d-f}$, $p=NS^{d-e,e-f}$

* The comparison of variables between the two hormonal treatments at all study points showed no statistically significant differences (NS)

with endometriosis [30]. The main effect of hormonal treatment occurs through the blockage of the hypothalamus–pituitary–ovary axis or by inducing pseudodecidualization leading to an amenorrheic status that impairs the progression of endometriosis implants [31]. Thus, hormonal treatments address these phenomena leading to a reduction of ectopic endometrial cell implantation, increasing apoptosis, and, as a whole, reducing inflammatory status [32].

Among the therapeutic armamentarium available for endometriosis, the most frequently therapies used are different combinations of estroprogestins and progestins, which have shown to have clinically equivalent actions and depend on the clinical profile of each patient [22–34]. Several studies have reported a reduction in the expression of the disease, even in DE lesions, with the use of hormonal treatments, inhibiting cyclic bleeding of endometriosis lesions [31–33, 35].

Dienogest is a 19-nortestosterone derivative and a selective progesterone receptor agonist that is widely used to treat endometriosis [33, 35]. The antiestrogenic effect of this steroid hormone allows it the ability to induce endometrial pseudodecidualization, diminish the inflammatory ambience and reduce oxidative stress [37, 38]. Several in vitro studies have demonstrated the increase of apoptosis induced by dienogest in endometriosis lesions in animal models and human endometriosis, specifically in stromal cells [39, 40]. This enhanced apoptosis in patients receiving dienogest treatment may explain the increase of cfDNA levels shown in our study.

Oral estroprogestin contraceptives prescribed in a flexible extended regimen have classically been used as empirical treatment in women with suspected endometriosis, being effective in reducing endometriosis symptoms [41, 42] as well as menstrual flow, causing decidualization of endometrial cells and leading to enhanced apoptosis of endometrial tissue and suppression of cell proliferation [43, 44]. Therefore, and similarly to dienogest, the increase in cell apoptosis in ectopic endometrium induced by estroprogestins may justify the higher levels of cfDNA found in DE patients receiving 2 mg dienogest/30 µg ethinylestradiol.

The above notwithstanding, rather than a definitive explanation and without direct evidence, our main hypothesis is that the increase in cfDNA levels may be explained by the pro-apoptotic effects triggered by the hormonal treatments and also the anti-angiogenic and anti-inflammatory properties of these hormonal treatments on endometriosis lesions. In previous publications dienogest, a molecule present in both hormonal treatment administered, has proven to reduce inflammation and angiogenesis involved in endometriosis lesions both in vitro and in vivo. Dienogest influences the inflammatory response in endometrial tissue through the modulation of prostaglandins, pro-inflammatory cytokines, interleukin (IL)-1b, IL-6, IL-8, tumor necrosis factor-alfa and growth factor biosynthesis (vascular endothelial growth factor and nerve growth factor), which are responsible for the control of inflammation [45]. Furthermore, in an experimental study in rats with induced endometriosis, dienogest proved to be an oral antagonist of angiogenesis. This antiangiogenic action could lead to a reduction of lesions and an increased apoptosis which could explain the surge in cfDNA levels [46]. Other previous research also evaluated the effects of dienogest in vivo by analyzing endometrioma tissue from patients exposed to dienogest compared to those who were not receiving any treatment. Cell proliferation, aromatase expression and blood vessel density were shown to be lower in the dienogest group. Furthermore, the TUNEL assay was used to detect apoptosis and the number of TUNEL-positive cells was higher in the dienogest group. These histologic events can explain the therapeutic effect of dienogest on endometriosis lesions and potentially explain the increased levels of cfDNA found in our study [40]. Combined oral contraceptives, have also proven to increase apoptosis in endometrial tissue by regulating cell growth. This was histologically analyzed in biopsy specimens of eutopic endometrium by taking a sample before and 30 days after the initiation of combined contraceptives. In this study, treatment with combined contraceptives showed a down-regulation of cell proliferation with a decrease of ki-76 expression and a pro-apoptotic effect with an increase of apoptotic cells [43]. These findings were also supported by a randomized control trial which studied the effect of combined oral contraception versus progestin-only treatment on cell proliferation and apoptosis of ectopic endometrial lesions. It concluded that

progestins and combined contraception had a pro-apoptotic effect on endometriosis lesions which was enhanced by the presence of ethinylestradiol in the group receiving combined contraception [44].

Estrogen dependency and progesterone resistance are endocrinologically known phenomena which initiate and maintain endometriosis lesions. This hormonal ambience enables ectopic implantation of endometrial cells, decreases apoptosis and increases inflammation. The hormonal treatments prescribed to patients aim to tackle these pathological endocrine aspects and have been described to increase apoptosis and reduce inflammation. In the literature, several studies have reported the pro-apoptotic effect of hormonal treatment in endometriosis. Some have even described a stronger effect when ethinylestradiol and progestogens are used simultaneously in combined contraception regimens [44]. Scarce information is available concerning an explanation for the differences in combined or progestin-only treatment regarding apoptosis. A previous study comparing cell growth of epithelial cells derived from endometriomas reported a suppression of cell growth under exposure to norethindrone or levonorgestrel, which was enhanced when adding ethinylestradiol. The exact mechanism behind this phenomenon is still uncertain, although it may involve an upregulation of progesterone receptor type B [47]. This finding was not observed in our study in which treatment with dienogest or dienogest/ethinylestradiol showed similarly increased cfDNA levels at 12 months of follow-up.

This study has several limitations, which should be considered for data analysis. First, a relatively small number of patients were included for analysis and the sample size was arbitrarily decided. Although arbitrary sample size determination is common in exploratory studies it undermines the robustness of the conclusions. Moreover, although the study was prospective, it was neither randomized nor blinded, which may introduce bias and limit the generalizability of the findings. Second, only one type of estroprogestin and progestin was evaluated and, therefore, differences among other types and combinations need to be evaluated in randomized studies. Nevertheless, although it may be a limitation to be considered when interpreting the results, our results are a real-life reflection of daily practice of hormonal treatment prescription in patients with DE. Third, we evaluated patients that underwent treatment for a long period due to good clinical response, and the few patients who stopped the treatment prematurely were excluded from the study. This may induce a possible selection bias that may limit the generalizability of the results. Fourth, we focused our study on DE patients, the most severe form of endometriosis and probably the main challenge in medical treatment of endometriosis. Furthermore, we did not design a study with controls with other forms of the disease or healthy control and these weaknesses of our research limit the generalizability of the findings. Fifth, we did not evaluate the levels of other apoptosis-associated markers that would be useful to understand our findings. Sixth, based on our results, cfDNA does not appear to be a reliable parameter for differentiating between elevation caused by the progression of endometriosis activity and elevation resulting from treatment response. This conclusion is drawn from the fact that, in this study, treatment response was assessed solely through clinical evaluation. Lastly, our follow-up was of up to 12 months, with no further data of what the long-term impact of these hormonal treatments may be.

The main strength of our study is the good characterization of endometriosis in all the patients by gynecological experts and avoidance of self-reported diagnosis. Finally, the long-term follow-up is of note since most endometriosis studies usually do not exceed 6 months.

This novel work opens the door to further research which should consider a longer follow-up and a larger sample size, as well as patients with different types of endometriosis and healthy controls to further elucidate if cfDNA could be used as a response or prognostic marker and help to understand the impact hormonal treatment has on endometriosis. Moreover, further studies should consider the evaluation of larger cohorts that will allow a more detailed subgroup analyses, such as disease severity or treatment duration, include randomization and treatment blinding and the addition of other hormonal treatments with different routes of administration and including responders and non-responders to specific treatments. Furthermore, future studies should include the analysis of other apotosis-associated markers, such as cytokeratins or cytochrome C in plasma, and assess the correlation with cfDNA levels. Finally, future studies involving objective measures of response, such as imaging alongside clinical evaluation, and other biomarkers in endometriosis (e.g., inflammatory cytokines or miRNAs) as well as the measurement of cfDNA through other complementary molecular mechanisms that could provide a more comprehensive picture of treatment effects are warranted to achieve further conclusions.

Conclusion

DE patients receiving oral first-line hormonal treatment showed higher cfDNA levels at 12 months of follow-up compared to baseline, with no differences according to the type of estroprogestin or progestin hormonal treatment prescribed.

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Author contributions
P.C.T.: investigation, writing; D.T.: conceptualization, resources, investigation, writing; H.C.: investigation; M.G.: investigation; G.F.: investigation; J.C.R.: conceptualization, resources, investigation, writing; F.C.: funding acquisition, investigation, writing; M.A.M.-Z.: conceptualization, funding acquisition, resources, investigation, writing. All authors have read and agreed to the published version of the manuscript.

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Availability of data and materials
No datasets were generated or analysed during the current study.

Declarations

Ethics approval and consent to participate
The study was conducted in accordance with the Declaration of Helsinki, and approved by the Ethics Committee of the Hospital Clínic of Barcelona (protocol code HCB/2020/1445 and date of approval 14 June 2021). Informed consent was obtained from all subjects involved in the study.

Consent for publication
Not applicable.

Competing interests
The authors declare no competing interests.

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References

1. Chapron C, Marcellin L, Borghese B, Santulli P. Rethinking mechanisms, diagnosis and management of endometriosis. Nat Rev Endocrinol. 2019;15:666–82.
2. Buck Louis GM, Hediger ML, Peterson CM, Croughan M, Sundaram R, Stanford J, et al. Incidence of endometriosis by study population and diagnostic method: the ENDO study. Fertil Steril. 2011;96:360–5.
3. Laganà AS, Garzon S, Götte M, Viganò P, Franchi M, Ghezzi F, et al. The pathogenesis of endometriosis: molecular and cell biology insights. Int J Mol Sci. 2019;20:5615.
4. Fassbender A, Vodolazkaia A, Saunders P, Lebovic D, Waelkens E, De Moor B, et al. Biomarkers of endometriosis. Fertil Steril. 2013;99:1135–45.
5. Nisenblat V, Bossuyt PM, Shaikh R, Farquhar C, Jordan V, Scheffers CS, et al. Blood biomarkers for the non-invasive diagnosis of endometriosis. Cochrane Database Syst Rev. 2016;2016(5): CD012179.
6. Collie B, Troisi J, Lombardi M, Symes S, Richards S. The current applications of metabolomics in understanding endometriosis: a systematic review. Metabolites. 2025;15(1):50.
7. Angioni S, Saponara S, Succu AG, Sigilli M, Scicchitano F, D'Alterio MN. Metabolomic characteristics in endometriosis patients. Springer; 2021. p. 9–17.
8. Jahr S, Hentze H, Englisch S, Hardt D, Fackelmayer FO, Hesch RD, Knippers R. DNA fragments in the blood plasma of cancer patients: quantitations and evidence for their origin from apoptotic and necrotic cells. Cancer Res. 2001;61:1659–65.
9. Song P, Wu LR, Yan YH, Zhang JX, Chu T, Kwong LN, et al. Limitations and opportunities of technologies for the analysis of cell-free DNA in cancer diagnostics. Nat Biomed Eng. 2022;6:232–45.
10. Galeazzi M, Morozzi G, Piccini M, Chen J, Bellisai F, Fineschi S, Marcolongo R. Dosage and characterization of circulating DNA: present usage and possible applications in systemic autoimmune disorders. Autoimmun Rev. 2003;2:50–5.
11. Zhong XY, von Mühlenen I, Li Y, Kang A, Gupta AK, Tyndall A, Holzgreve W, Hahn S, Hasler P. Increased concentrations of antibody-bound circulatory cell-free DNA in rheumatoid arthritis. Clin Chem. 2007;53:1609–14.
12. Lo YM, Rainer TH, Chan LY, Hjelm NM, Cocks RA. Plasma DNA as a prognostic marker in trauma patients. Clin Chem. 2000;46:319–23.
13. Hao TB, Shi W, Shen XJ, Qi J, Wu XH, Wu Y, Tang YY, Ju SQ. Circulating cell-free DNA in serum as a biomarker for diagnosis and prognostic prediction of colorectal cancer. Br J Cancer. 2014;111:1482–9.
14. Zachariah R, Schmid S, Radpour R, Buerki N, Fan AXC, Hahn S, et al. Circulating cell-free DNA as a potential biomarker for minimal and mild endometriosis. Reprod Biomed Online. 2009;18:407–11.
15. Alonso A, Yuwono NL, Houshdaran S, Abbott J, Rodgers R, Ford CE, et al. Comparison of total and endometrial circulating cell-free DNA in women with and without endometriosis. Reprod Biomed Online. 2022;44:495–503.
16. Yuwono NL, Alonso A, Abbott J, Houshdaran S, Henry CE, Rodgers R, et al. Circulating cell-free endometrial DNA level is unaltered during menstruation and in endometriosis. Hum Reprod. 2022;37:2560–9.
17. Wang W, Kong P, Ma G, Li L, Zhu J, Xia T, Xie H, Zhou W, Wang S. Characterization of the release and biological significance of cell-free DNA from breast cancer cell lines. Oncotarget. 2017;8:43180–91.
18. Jorch SK, Kubes P. An emerging role for neutrophil extracellular traps in noninfectious disease. Nat Med. 2017;23:279–87.
19. Pinegin B, Vorobjeva N, Pinegin V. Neutrophil extracellular traps and their role in the development of chronic inflammation and autoimmunity. Autoimmun Rev. 2015;14:633–40.
20. Berkes E, Oehmke F, Tinneberg HR, Preissner KT, Saffarzadeh M. Association of neutrophil extracellular traps with endometriosis-related chronic inflammation. Eur J Obstet Gynecol Reprod Biol. 2014;183:193–200.
21. Munrós J, Tàssies D, Reverter JC, Martin L, Pérez A, Carmona F, et al. Circulating neutrophil extracellular traps are elevated in patients with deep infiltrating endometriosis. Reprod Sci. 2019;26:70–6.
22. Capezzuoli T, Rossi M, La Torre F, Vannuccini S, Petraglia F. Hormonal drugs for the treatment of endometriosis. Curr Opin Pharmacol. 2022;67:102311.
23. Wiegratz I, Kuhl H. Long-cycle treatment with oral contraceptives. Drugs. 2004;64:2447–62.
24. Klipping C, Duijkers I, Fortier MP, Marr J, Trummer D, Elliesen J. Contraceptive efficacy and tolerability of ethinylestradiol 20 µg/drospirenone 3 mg in a flexible extended regimen: an open-label, multicentre, randomised, controlled study. J Family Plan Reprod Health Care. 2012;38:73–83.
25. Mitchell JB, Chetty S, Kathrada F. Progestins in the symptomatic management of endometriosis: a meta-analysis on their effectiveness and safety. BMC Womens Health. 2022;22:526.
26. Guerriero S, Condous G, van den Bosch T, Valentin L, Leone FPG, Van Schoubroeck D, et al. Systematic approach to sonographic evaluation of the pelvis in women with suspected endometriosis, including terms, definitions and measurements: a consensus opinion from the International Deep Endometriosis Analysis (IDEA) group. Ultrasound Obstet Gynecol. 2016;48:318–32.
27. van den Bosch T, Dueholm M, Leone FPG, Valentin L, Rasmussen CK, Votino A, et al. Terms, definitions and measurements to describe sonographic features of myometrium and uterine masses: a consensus opinion from the Morphological Uterus Sonographic Assessment (MUSA) group. Ultrasound Obstet Gynecol. 2015;46:284–98.
28. Ros C, Rius M, Abrao MS, deGuirior C, Martínez-Zamora MÁ, Gracia M, Carmona F. Bowel preparation prior to transvaginal ultrasound improves detection of rectosigmoid deep infiltrating endometriosis and is well tolerated: prospective study of women with suspected endometriosis without surgical criteria. Ultrasound Obstet Gynecol. 2021;57:335–41.
29. Carrillo Torres P, Martínez-Zamora MÁ, Ros C, Rius M, Mensión E, Gracia M, et al. Clinical and sonographic impact of oral contraception in patients with deep endometriosis and adenomyosis at 2 years of follow-up. Sci Rep. 2023;13:2066.
30. Becker CM, Bokor A, Heikinheimo O, Horne A, Jansen F, Kiesel L, et al. ESHRE guideline: endometriosis. Hum Reprod Open. 2022. https://doi.org/10.1093/hropen/hoac009.
31. Vannuccini S, Clemenza S, Rossi M, Petraglia F. Hormonal treatments for endometriosis: The endocrine background. Rev Endocr Metab Disord. 2022;23:333–55.

32. Patel BG, Lenk EE, Lebovic DI, Shu Y, Yu J, Taylor RN. Pathogenesis of endometriosis: interaction between Endocrine and inflammatory pathways. Best Pract Res Clin Obstet Gynaecol. 2018;50:50–60.
33. El Taha L, Abu Musa A, Khalifeh D, Khalil A, Abbasi S, Nassif J. Efficacy of dienogest vs combined oral contraceptive on pain associated with endometriosis: randomized clinical trial. Eur J Obstet Gynecol Reprod Biol. 2021;267:205–12.
34. Vercellini P, Buggio L, Berlanda N, Barbara G, Somigliana E, Bosari S. Estrogen-progestins and progestins for the management of endometriosis. Fertil Steril. 2016;106:1552-1571.e2.
35. Momoeda M, Harada T, Terakawa N, Aso T, Fukunaga M, Hagino H, et al. Long-term use of dienogest for the treatment of endometriosis. J Obstet Gynaecol Res. 2009;35:1069–76.
36. Ferrero S, Venturini PL, Remorgida V. Changes in the size of rectovaginal endometriotic nodules infiltrating the rectum during hormonal therapies. Fertil Steril. 2010;94:S39.
37. Barra F, Scala C, Ferrero S. Current understanding on pharmacokinetics, clinical efficacy and safety of progestins for treating pain associated to endometriosis. Expert Opin Drug Metab Toxicol. 2018;14:399–415.
38. Reis FM, Petraglia F, Taylor RN. Endometriosis: hormone regulation and clinical consequences of chemotaxis and apoptosis. Hum Reprod Update. 2013;19:406–18.
39. Choi J, Jo M, Lee E, Lee DY, Choi D. Dienogest regulates apoptosis, proliferation, and invasiveness of endometriotic cyst stromal cells via endoplasmic reticulum stress induction. Mol Hum Reprod. 2020;26:30–9.
40. Miyashita M, Koga K, Takamura M, Izumi G, Nagai M, Harada M, Hirata T, Hirota Y, Fujii T, Osuga Y. Dienogest reduces proliferation, aromatase expression and angiogenesis, and increases apoptosis in human endometriosis. Gynecol Endocrinol. 2014;30:644–8.
41. Harada T, Kosaka S, Elliesen J, Yasuda M, Ito M, Momoeda M. Ethinylestradiol 20 µg/drospirenone 3 mg in a flexible extended regimen for the management of endometriosis-associated pelvic pain: a randomized controlled trial. Fertil Steril. 2017;108:798–805.
42. Harada T, Momoeda M, Taketani Y, Hoshiai H, Terakawa N. Low-dose oral contraceptive pill for dysmenorrhea associated with endometriosis: a placebo-controlled, double-blind, randomized trial. Fertil Steril. 2008;90:1583–8.
43. Meresman GF, Augé L, Barañao RI, Lombardi E, Tesone M, Sueldo C. Oral contraceptives suppress cell proliferation and enhance apoptosis of eutopic endometrial tissue from patients with endometriosis. Fertil Steril. 2002;77:1141–7.
44. Sophonsritsuk A, Tantanavipas S, Tingthanatikul Y, Lertvikool S, Dittharot K, Waiyaput W. Effects of ethinyl estradiol in combined oral contraceptives on cell proliferation and apoptosis in ectopic endometrial tissue: a randomized controlled study. J Family Reprod Health. 2021;15:45–52.
45. Grandi G, Mueller M, Bersinger NA, Cagnacci A, Volpe A, McKinnon B. Does dienogest influence the inflammatory response of endometriotic cells? A systematic review. Inflamm Res. 2016;65(3):183–92.
46. Nakamura M, Katsuki Y, Shibutani Y, Oikawa T. Dienogest, a synthetic steroid, suppresses both embryonic and tumor-cell-induced angiogenesis. Eur J Pharmacol. 1999;386(1):33–40.
47. Bono Y, Kyo S, Kiyono T, Mizumoto Y, Nakamura M, Maida Y, et al. Concurrent estrogen action was essential for maximal progestin effect in oral contraceptives. Fertil Steril. 2014;101(5):1337–43.

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