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human reproduction **ORIGINAL ARTICLE** ***Gynaecology***

Dienogest mediates midkine suppression in endometriosis

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STUDY QUESTION: What are the effects of dienogest (DNG) on midkine (MK) production in women with endometriosis?

SUMMARY ANSWER: DNG-mediated down-regulation of MK *in vivo* and *in vitro*.

WHAT IS KNOWN ALREADY: DNG is an oral progestin that alleviates painful symptoms of women with endometriosis with a favourable tolerability and safety profile. Its effects on MK, a growth factor that plays an important role in endometriosis, have not yet been investigated.

STUDY DESIGN, SIZE, DURATION: Prospective *in vivo* study on 283 patients subjected to laparoscopy for benign pathologies in a University hospital and *in vitro* cultures of primary endometrial stromal cells (ESC) from 6 of these women with histologically confirmed endometriosis.

PARTICIPANTS/MATERIALS, SETTING, METHODS: MK concentrations in the peritoneal fluid (PF) of women were measured by ELISA and compared based on endometriosis status and the use of DNG. A subsequent *in vitro* analysis with ESC was used to confirm the direct influence of DNG and other progestins including, norethisterone acetate (NETA) and medroxyprogesterone acetate (MPA) on MK mRNA production.

MAIN RESULTS AND THE ROLE OF CHANCE: The final study population consisted of 253 women. Of these, 165 suffered from endometriosis, with 62 of them taking DNG (DNG group) and 103 taking no hormone treatment (non-DNG group) during at least 3 months before surgery. Another 88 women were endometriosis free (non-endometriosis group). The concentration of MK was highest in the PF of women in the non-DNG group (median 5.26 ng/ml, IQR 2.74–8.46). Significantly lower concentrations were found in the non-endometriosis group (median 3.51 ng/ml, IQR: 1.90–7.53, $P = 0.028$). The lowest concentrations were found in the DNG group (median 2.44 ng/ml, IQR: 1.12–4.70, $P < 0.0001$ versus non-DNG group, $P = 0.048$ versus non-endometriosis group). The treatment of primary cultured ESC with DNG (10^{-5} M) suppressed MK mRNA production ($P = 0.016$), whereas MPA ($P = 0.109$) and NETA ($P = 0.422$) at same concentrations did not show a similar effect.

LIMITATIONS, REASONS FOR CAUTION: The non-randomized design of the study.

WIDER IMPLICATIONS OF THE FINDINGS: These findings could indicate a direct effect of DNG on endometriotic cells that could contribute to its effectiveness in the treatment of this disease.

STUDY FUNDING/COMPETING INTEREST(S): Funding was received from Swiss National Science Foundation (Grant No. 320030_140774). M.D.M. has received fees for speaking at scientific meetings from Bayer. The other authors have no conflicts of interest to declare.

The authors state that the manufacturer of dienogest has in no way influenced the performance or outcomes of this study.

Key words: endometriosis / midkine / growth factors / cytokines / dienogest / norethisterone acetate / norethindrone acetate / medroxyprogesterone acetate

Introduction

Endometriosis is a prevalent gynecological disorder affecting at least 10% of reproductive-aged women worldwide (Eskenazi and Warner, 1997). It is characterized by the growth of endometrial epithelial and stromal cells outside the uterine cavity and can result in severe pelvic pain and subfertility. In addition, it is increasingly being recognized as an inflammatory disease with elevated concentrations of growth

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factors and cytokines in the peritoneal fluid (PF) of women with endometriosis compared with women without (Koga et al., 2000; Osuga et al., 2000; Yoshino et al., 2003; Bersinger et al., 2006). Local inflammatory and immunological phenomena (Sturlese et al., 2011; Králíčková and Vetvicka, 2015; Salmeri et al., 2015; Laganà et al., 2016) create this altered peritoneal microenvironment that facilitates the pathogenesis of endometriosis and may explain why some women experience more severe symptoms than others (McKinnon et al., 2015).

Midkine (MK) is a low-molecular weight non-glycosylated protein also known as neurite growth-promoting factor-2. It is highly expressed during the midgestation stage of embryogenesis, but is restricted to a limited number of tissue in adults (Muramatsu, 2011). There is also a strong induction of MK during oncogenesis, inflammation and tissue repair (Muramatsu, 2002) making MK a protein of interest in disease progression and pharmaceutical targeting. MK may contribute to the pathogenesis of endometriosis through the stimulation of cell proliferation, migration, angiogenesis and fibrinolysis (Muramatsu, 2010), as well as to neuronal growth (Winkler and Yao, 2014). Higher expression of MK in the endometrium of women with endometriosis compared with women without has been confirmed (Chung et al., 2002). PF concentrations of MK are higher in women with advanced stages of the disease and MK stimulates the proliferation of cultured endometrial stromal cells (ESC; Hirota et al., 2005).

The suppression of MK by gonadotrophin-releasing hormone analogues (GnRHa), which have been used as a first line treatment for endometriosis-related pain may be an important facet in the efficacy of these drugs (Hirota et al., 2005; Nirgianakis et al., 2013). More recently, however, dienogest (DNG), a synthetic progestin, has been introduced for the treatment of endometriosis providing similar efficacy, but better tolerability than GnRHa (Cosson et al., 2002; Strowitzki et al., 2010). Whether DNG also influences MK concentrations has not yet been investigated, although direct anti-proliferative and anti-inflammatory effects have been observed on endometriotic stromal cells (Horie et al., 2005; Fu et al., 2008; McCormack, 2010; Grandi et al., 2016).

In the present study, we therefore wished to further examine the effects of DNG through investigating its impact on MK production. To do this, we evaluated the influence of DNG on MK PF concentrations and performed subsequent *in vitro* experiments to determine if the influence of DNG occurs directly on endometrial tissue.

Materials and Methods

Patient selection and collection of PF samples

This study was prepared according to the 'Strengthening the Reporting of Observational studies in Epidemiology' guidelines (von Elm et al., 2007). Women undergoing laparoscopic surgery for benign pathologies in the Department of Obstetrics and Gynaecology, University of Berne (Switzerland) between June 2011 and October 2015 were included in the study. Exclusion criteria were the use of hormonal drugs other than DNG 2 mg daily (Visanne®, Bayer Healthcare, Leverkusen, Germany) in the 3 months prior to surgery, patients suffering from other inflammatory diseases, pregnancy, malignancy and surgery performed in an emergency situation. Written informed consent and detailed information on hormonal treatment usage was obtained from all participants prior to surgery, and the project was approved by the relevant Ethical committee. All procedures were performed during the proliferative phase of the menstrual cycle, confirmed by the measurement of progesterone in the PF with a cut-off value of 10 nmol/l. Intraoperative findings and revised American Fertility Society stage of endometriosis were documented (rAFS, 1997).

Eutopic endometrium was obtained via endometrial Pipelle® (Pipelle deCornier, Laboratoire CCD, Paris, France) biopsies and the PF collected from the cul-de-sac at the beginning of the laparoscopy before any other surgical procedure had been performed. The endometrial biopsies were transferred to culture medium containing fetal calf serum (10%, v/v) and DMSO (10–20%, v/v), slow-frozen to −80°C in a Bicell® chamber and stored in liquid nitrogen for later use. PF samples were clarified by centrifugation (10 min at 1800 × *g*) and stored undiluted in 0.5–1.0-ml aliquots at −70°C prior to assay. Cases with haemolysed PF supernatants or insufficient PF volume were excluded, as described previously (McKinnon et al., 2014). For comparison of MK concentrations, patients were split into three groups: (i) patients with endometriosis receiving DNG prior to surgery (DNG group), (ii) patients with endometriosis but no hormonal treatment prior to surgery (non-DNG group) and (iii) patients without endometriosis (non-endometriosis group).

MK measurement in PF

The total protein concentration in the PF samples was determined with the micro-bicinchoninic acid method (Quanti-Pro® BCA, Sigma-Aldrich, St. Louis, MO, USA; Nisolle et al., 1994). MK concentrations were determined by microplate ELISA as described in the previous reports from our laboratory (Bersinger et al., 2006; Scholl et al., 2009). At the time of these measurements the laboratory had no knowledge of the presence or the absence of endometriosis or of DNG treatment.

Isolation and culture of primary ESC

Primary ESC were isolated from endometrial biopsies from six different women with histologically confirmed endometriosis and no hormonal therapies within the previous 3 months, using methods described previously (McKinnon et al., 2014). Briefly, separation was performed via collagenase digestion (Collagenase from *Clostridium histolyticum*, Sigma Life Sciences, MO, USA) and size exclusion membranes (100 and 40 μm mesh filters, BD Bioscience, NJ, USA). Isolated ESC were maintained in Iscoves's modified Eagle medium (IMEM) (Invitrogen Life Technologies, NY, USA) supplemented with 10% (v/v) fetal calf serum (FCS) and 1% (v/v) antibiotic/antimycotic (Invitrogen Life Technologies) at 37°C in a humidified atmosphere. CO_2 was added to the normal atmospheric conditions. The culture medium was changed every 3 days.

In the experiments cells were seeded onto 6-well plates and grown until ~80% confluence. Prior to treatments the FCS concentration in the medium was changed to 0.5% (v/v). *In vitro* treatments were performed for 6 h with medroxyprogesterone acetate (MPA; Sigma-Aldrich), norethisterone acetate (NETA; Sigma-Aldrich) and DNG (Bayer HealthCare, Leverkusen, Germany), all at a concentration of 10^{-5} M. After incubation, the cells were collected in RNA lysis buffer (Qiazol® Lysis Reagent, Qiagen, MD, USA).

Analysis of MK gene expression

RNA extraction was performed with the RNAeasy® Plus Micro extraction Kit (Qiagen) following the manufacturer's instructions. One microgram of RNA was reverse transcribed to cDNA in a final volume of 25 μl with the Moloney Murine leukaemia virus Reverse transcriptase enzyme and random primers (Promega, Madison, WI, USA) and diluted 1:20. Genomic DNA absence was confirmed via a no-enzyme RT control (RT-). Gene expression was determined by Real-time quantitative Polymerase chain reaction (qPCR) using Rotor-gene Taqman Fast advanced Master Mix (Qiagen) and the following TaqMan® gene expression primer/probes for MK gene (Hs00171064_m1) and reference genes hypoxanthine phosphoribosyltransferase-1 (HPRT1; Hs01003267_m1), beta-actin (Hs01060665_g1), ubiquitin C (Hs00824723_m1), glyceraldehyde

3-phosphate dehydrogenase (GAPDH; Hs00266705_g1) and ribosomal protein L13A (RPL13A; Hs04194366_g1). The qPCR was performed in a Rotor-Gene RG 2000 (Corbett Research, New South Wales, Australia), under the following conditions: 95°C for 5 min, followed by 40 cycles consisting of 95°C for 5 s and 60°C for 10 s.

Reference genes were selected with the qBASE software suite based on their stability across samples (Biogazelle, Ghent University, Belgium) and the change in mRNA expression for MK calculated via the qBASE software, based on the geometric mean of the multiple reference genes and the ΔΔCt method. The efficiency of each reaction, as determined via linear regression (Kato *et al.*, 2000) was also incorporated into the equation. All RNA quantities are expressed as percentages of control.

Statistical analysis

Median values and interquartile range (IQR) or mean and standard deviation were calculated for continuous variables as appropriate and percentages for the qualitative variables. Statistical analysis for the comparison of MK concentrations between groups was done by Mann–Whitney *U* test, while for MK mRNA production a non-parametric Wilcoxon matched paired test was used. Other parameters (age, BMI, protein concentration, volume of aspirated PF) were compared between groups by χ^2 or ANOVA as appropriate. Graph-Pad Prism version 3.03 was used, and the significance was set at a *P* value of <0.05.

Results

A total of 283 patients were included in the study. In 30 patients it was not possible to measure the PF MK concentration due either to a dilution of the fluid under the surgical procedure (detected by the aspect and a low total protein concentration) or to the presence of haemolysis; these cases were thus excluded from the statistical analysis. In the remaining 253 patients, 165 (65.2%) were diagnosed with endometriosis, with 62 (24.5%) using DNG (DNG group) and 103 (40.7%) using no hormonal treatments (non-DNG group). The remaining 88 (34.8%) patients were confirmed endometriosis free and were not taking any hormonal treatments prior to surgery (non-endometriosis group). A comparison between groups showed that the stage of endometriosis, as measured by the revised American Fertility Society (rAFS) staging system, was significantly higher in the DNG than in the non-DNG group ($P = 0.0405$). Patient characteristics and PF concentrations are shown in Table I.

MK concentrations in PF

MK concentrations in the PF of women with endometriosis were highest in the non-treated endometriosis (non-DNG) group (median 5.26, IQR 2.74–8.46 ng/ml). Compared with that, MK concentrations were significantly lower in both the non-endometriosis group (median 3.51, IQR: 1.90–7.53 ng/ml, $P = 0.0275$) and the DNG-treated endometriosis group (median 2.44, IQR: 1.12–4.70 ng/ml, $P < 0.0001$). MK concentrations in the DNG-treated endometriosis group were even significantly lower than in the group without endometriosis ($P = 0.0483$) (Fig. 1).

Inhibition of MK mRNA production

A significant reduction in MK mRNA production when compared with control (100%) was observed after treatment with 10^{-5} M DNG (36.31 ± 25.20%, $P = 0.016$) (Fig. 2A). In contrast, a non-significant increase in MK expression was observed after treatment with 10^{-5} M MPA (172.40 ± 115.00%, $P = 0.109$) (Fig. 2B) and 10^{-5} NETA (180.80 ± 168%, $P = 0.422$) (Fig. 2C).

Discussion

In the present study, we were able to demonstrate that DNG suppressed MK concentrations in the PF of women with endometriosis. Moreover, MK levels were lower in women with endometriosis under DNG even though they presented with a higher rAFS stage than women without DNG. This influence was confirmed in an *in vitro* treatment of primary ESC from endometriotic women at the mRNA level and it was specific to DNG as other progestins used for the treatment of endometriosis failed to produce a similar effect.

MK is a potent proliferative and neurogenic mediator that enhances the angiogenic and proliferative activities of cancer cells (Kato *et al.*, 2000). The expression of MK is elevated in multiple cancers, such as neuroblastoma, glioblastoma, Wilms' tumours, thyroid papillary

Table I Patient characteristics in the three groups of patients included.

	Endometriosis		Non-endometriosis group	*P* Value
	DNG group	Non-DNG group		
Number of women	62	103	88	
Age (years)	31.1 ± 5.7	34.4 ± 5.9	36.4 ± 7.9	**<0.0001**[a]
Body mass index (kg/m^2)	22.9 ± 3.4	23.5 ± 4.4	24.4 ± 4.1	0.0700[a]
rAFS stage I	15 (24)	32 (31)	n/a	**0.0405**[b]
rAFS stage II	6 (10)	17 (17)	n/a	
rAFS stage III	8 (13)	22 (21)	n/a	
rAFS stage IV	33 (53)	32 (31)	n/a	
PF collected (ml)	8.8 ± 8.2	10.7 ± 9.0	8.7 ± 6.6	0.1586[a]
Protein concentration in PF (mg/ml)	38.0 ± 12.1	33.5 ± 14.5	35.8 ± 12.1	0.0991[a]

Data are presented as *n* (%), or mean ± SD. Significant *P*-values are marked bold.
DNG, dienogest; rAFS, revised American Fertility Society; PF, peritoneal fluid.
[a]Statistical analysis performed by one-way ANOVA.
[b]Statistical analysis performed by χ^2 in a 2 × 4 contingency table.

carcinomas, colorectal, liver, ovary, bladder, breast, lung, oesophageal, stomach and prostate cancers (Ikematsu *et al.*, 2000). It has also been found elevated in the PF of women with endometriosis (Chung *et al.*, 2002; Hirota *et al.*, 2005), a result we were able to confirm. In contrast, its expression in normal adult tissue is limited. Such an increase in benign and malignant tumours suggests an important role for MK in the pathogenesis of these conditions. MK has also been shown to activate the AKT/mTOR pathway leading to increased cellular proliferation and cell resistance to chemotherapeutics (Mirkin *et al.*, 2005) and this kinase signalling pathway may play an important role in endometriosis (McKinnon *et al.*, 2016). Moreover, as a neurite growth-promoting factor, MK could be an important molecule for peripheral nerve stimulation and sensitization in endometriosis, as described recently (McKinnon *et al.*, 2015). Finally, several studies have focused on the possible dissemination of primordial endometrial stem cells in ectopic regions during the organogenesis and differentiation of Müllerian structures of the female reproductive tract (Laganà *et al*, 2013; Kobayashi *et al.*, 2014). This could predispose to endometriosis in these regions later in life. Since MK is widely produced during the midgestation stage of embryogenesis it could be possible that it is implicated in this process.

The role of MK in pathogenesis and thus the local influence of DNG on endometriotic lesion growth and symptomatology is not answered by this study, although it deserves further attention. If the importance of MK in the establishment and development of endometriosis can be confirmed it may be interesting to explore the therapeutic potential of MK inhibitors, either in combination with progestins or alone, in the treatment of endometriosis.

At present only two progestins, NETA and MPA (Quaas *et al*, 2015), have been approved by the US Food and Drug Administration (http://www.fda.gov/Drugs) for the treatment of endometriosis. A third progestin, DNG, has received approval as a monotherapy for the treatment of endometriosis in Europe, Japan, Australia and Singapore (Dunselman *et al.*, 2014). Amongst these three progestins, only DNG decreased MK mRNA production by ESC in our *in vitro* study implying multiple mechanisms of action at the transcriptional level may exist for the different progestins.

Previous evidence supports a similar progesterone receptor (PR)-mediated activity of DNG and other progestins on protein production and secretion in ESC from women with endometriosis (Okada *et al.*, 2001; Horie *et al.*, 2005; McCormack, 2010; Grandi *et al.*, 2016). Paradoxically, however, ESC from women with endometriosis are considered progesterone resistant due to a significant down-regulation of the PR (Bulun *et al.*, 2010). Furthermore, the different progestins have varied affinities to other nuclear receptors, including the glucocorticoid, mineralocorticoid and androgen receptors that also stimulate gene transcription. A combination of the suppressed PR expression and various affinities for other nuclear receptors therefore could lead to variations in the transcriptional activities of these medications.

The *in vitro* effect of DNG on stromal cells could partially explain the *in vivo* DNG-mediated MK down-regulation observed here. However, it is not clear whether this additional mode of action provides a more effective treatment of endometriosis. A recent study showed that DNG was not associated with a statistically significant improvement in overall pain relief, psychological status, sexual functioning, or health-related quality of life if compared with NETA treatment (Vercellini *et al.*,

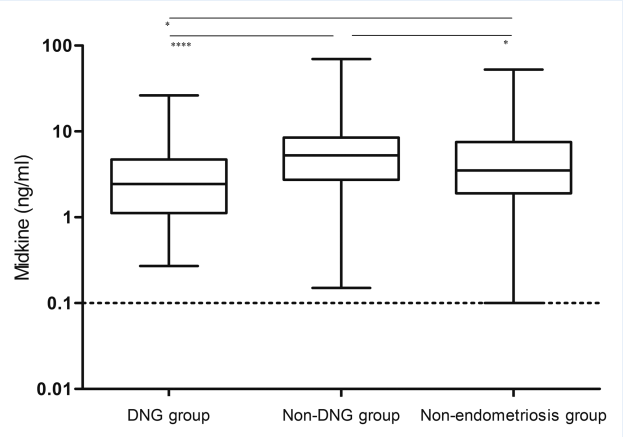

Figure 1 Peritoneal fluid (PF) midkine concentrations in the three groups of patients. Midkine (MK) concentrations in women with non-treated endometriosis (non- Dienogest (DNG) group; $n = 103$) were significantly higher than those in women without endometriosis (non-endometriosis group; $n = 88$). DNG treatment (DNG group; $n = 62$) suppressed PF MK concentrations to levels even below those observed in women without endometriosis ($^*P < 0.05$, $^{****}P < 0.0001$). Boxes represent the median with 25th and 75th percentiles, the whiskers the minimum and the maximum. The dotted line represents the detection limit of the assay.

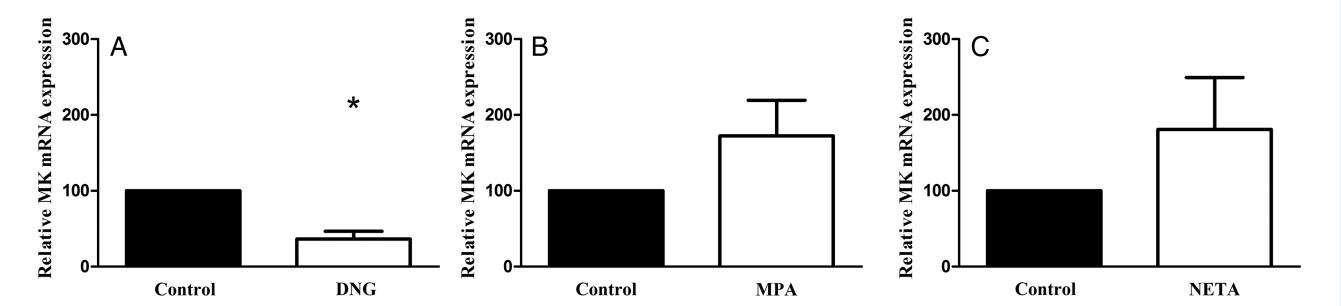

Figure 2 Midkine (MK) mRNA production (% of control) in cultured primary endometrial stromal cells (ESC; Mean + sem, $n = 6$) isolated from women with endometriosis. (**A**) Dienogest (DNG) treatment of primary ESC significantly suppressed MK production after 6 h of incubation ($^*P < 0.05$). In contrast both (**B**) medroxyprogesterone acetate (MPA) and (**C**) norethisterone acetate (NETA) non-significantly increased the production of MK mRNA.

2016). Moreover, it cannot be excluded that NETA and MPA could have also down-regulated MK *in vivo*. This could be possible through a mechanism of action other than the suppression of MK mRNA production by ESC observed with DNG. However, this was impossible to be evaluated in the current study since none of the patients was taking any of these two progestins.

In the present study, both rAFS stage and patient age varied significantly between the three groups of patients. A more advanced rAFS stage in the women using DNG is not surprising, given that these women are more likely to have suffered from severe pain and thus more likely to be prescribed a medical treatment. It would be expected that this increase in rAFS stage should be associated with an increase in the MK concentration, whereas due to the effect of DNG the opposite is observed. Women in the DNG group are also significantly younger than those in the other groups. However, it is unlikely that this could have influenced the results since in this case the highest MK concentrations would have been expected in the non-endometriosis group that actually includes the oldest patients. The main limitation of the current study, however, is its non-randomised design. On the other hand, MK concentrations were measured and documented prospectively with the laboratory assistants being blinded on clinical and surgical patient information.

In summary, the present study demonstrates for the first time a DNG-mediated *in vivo* and *in vitro* MK down-regulation in endometriosis. This effect could contribute to both the reduction of endometriotic lesion size and pain relief observed in patients with endometriosis after treatment with DNG.

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Authors' roles

K.N.: concept and design, study execution, data analysis, interpretation, manuscript draft, manuscript revise, final approval; G.G.: concept and design, study execution, data analysis, interpretation, manuscript draft, manuscript revise, final approval; B.Mc.K.: concept and design, manuscript draft, manuscript revise, final approval; N.A.B.: concept and design, study execution, data analysis, final approval; A.C.: concept and design, interpretation, final approval; M.D.M.: concept and design, interpretation, manuscript revise, final approval.

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Conflict of interest

In adherence to the conflict of interest policy recommended by the International Committee of Medical Journal Editors (ICMJE) K.N., G.G., B.Mc.K., N.A.B. and A.C. state explicitly that potential conflicts of interest do not exist for this research work. M.D.M. has received fees for speaking at scientific meetings from Bayer. The authors state that the manufacturer of dienogest has in no way influenced the performance or outcomes of this study.

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