

Full Length Research Paper

Effects of Metformin on Endocrine and Endometrial Expression of Androgen Receptors in Patients with Polycystic Ovary Syndrome

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Article history

Received: 15-10-2017

Revised: 25-10-2017

Accepted: 01-11-2017

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Abstract

This study was undertaken to evaluate the effects of metformin on endocrine and pattern of immunohistochemical expression of androgen receptors in endometrial tissue in patients with polycystic ovary syndrome. The study included 50 women with polycystic ovary syndrome (PCOS). Each patient was submitted to detailed history taking, clinical examination including hair all over the body, body weight, body height and body mass index (BMI), transvaginal sonography, laboratory investigations as: LH, FSH, fasting insulin, free testosterone. Endometrial samples were taken for dating and assessment of androgen receptors. These investigations were done before and after three months of metformin treatment. There was a significant decrease of BMI of the studied cases after metformin administration from 26.5 ± 0.6 kg/m² to 25.1 ± 0.39 kg/m². There were a significant decrease of LH level after metformin administration from 8.07 ± 2.48 mIU/ml to 6.85 ± 3.5 mIU/ml and a significant decrease of fasting insulin level from 13.5 ± 5.3 before metformin administration to 10.2 ± 6.9 after administration. Before metformin treatment, histopathological examination of the endometrial specimens revealed features of early proliferative endometrium in 32 cases, late proliferative in 10 cases, while the remaining 8 cases showed features of simple endometrial hyperplasia. After treatment, 30 cases out of the 32 cases with early proliferative endometrium progressed to late proliferative endometrium, while the remaining 2 cases showed no morphologic changes. The 10 cases with late proliferative endometrium progressed to early secretory (3 cases), mid-secretory (3 cases) and late secretory endometrium (4 cases) indicative of ovulation. Regression of hyperplasia was noted in 3 out of the 8 cases diagnosed as simple endometrial hyperplasia. As regard to immunohistochemistry (IHC), we found that women with PCOS exhibited marked increase in endometrial androgen receptors (AR) expression compared to normal fertile controls ($p < 0.001$). Significant decrease in AR expression in endometrial tissue after metformin treatment was observed. Metformin treatment decreases LH, fasting insulin, induces ovulation, decrease AR expression in endometrial tissue and restores menstrual cyclicity in patients with PCOS.

Keywords: PCOS, Metformin, Receptors, Polysystic, Syndrome.

Cite this article as: El Nagar, T.M., F. Midan, M., Rashed, R.M., Emran, T.M., & Semary, S.S. (2017). Effects of Metformin on Endocrine and Endometrial Expression of Androgen Receptors in Patients with Polycystic Ovary Syndrome. *Global Journal of Current Research*, 5(4), 187-192. Retrieved , from www.crdeepjournal.org/gjcr

Introduction

Polycystic ovary syndrome (PCOS) is a common disorder of women in reproductive age characterized by clinical and biochemical disorders and it's main symptoms might appear from adolescence. PCOS is characterized by menstrual disorders (amenorrhea or oligomenorrhea), anovulation, hyperandrogenism and polycystic change of ovaries. Insulin-resistant hyperinsulinism is an important extrinsic factor in the steroidogenic dysregulation of PCOS (Kang and Paek, 2017). Although obesity, insulin resistance, and hyperinsulinemia are not required for the diagnosis of PCOS, they commonly occur in women with PCOS, increasing susceptibility to metabolic complications such as type 2 diabetes, hyperlipidemia, hypertension, fatty liver and sleep apnea (Sirmans SM et al., 2013). Consequently, PCOS causes endocrine, metabolic, and cardiovascular symptoms in affected women. Women with PCOS usually seek treatment to manage the androgen-related symptoms, menstrual-related disorders, and infertility. (Saha et al., 2012). However, they also have various long-term complications which have been frequently underestimated. For instance, gestational diabetes and hypertensive disorders are more likely to occur in women with PCOS during

pregnancy, and as they get older, metabolic diseases including glucose intolerance, type 2 diabetes, or hyperlipidemia are frequently observed). Paige *et al.*, 2014 (Therefore, lifelong follow-up and management for these patients at risk are needed to detect and prevent complications as early as possible.

Metformin is an insulin-sensitizing drug that had demonstrated promise in therapy for patients with PCOS (Yashasvi *et al.*, 2016). Metformin treatment administered in high doses (3000 mg/day) on a 6 months period was useful for weight loss, improvement of ovarian function and decrease of androgen levels in obese PCOS patients (Aurelian and Anca, 2017). Metformin therapy to PCOS women lowered serum fasting insulin, total and free testosterone as well as estradiol levels at oocyte retrieval, enhanced clinical pregnancy and live birth rates, and diminished the risk of severe ovarian hyperstimulation syndrome (Kang and Paek, 2017). Metformin treatment may cause gastrointestinal side effects such as nausea, which often disappear with long-term use and it is rarely associated with lactic acidosis (Glintborg D *et al.*, 2014).

Androgen receptors (AR) have been identified in human endometrium, however, their role in endometrial cyclic development and function remains poorly understood (Apparao *et al.* 2002). Although the proliferation and differentiation of endometrium are mediated mainly by oestrogen and progesterone receptors, the androgen receptor may play some role in modulating these changes suggesting that AR may be involved in both physiological and pathological changes of the endometrium (Cloke B, Christian, 2012).

AR receptor content was seen in all cell types of the human endometrium and myometrium and was cyclic dependent. AR expression is primarily under androgenic control and high levels adversely affect priming of the endometrium in the follicular phase or development in the luteal phase (Shang *et al.* 2012).

Patients and Methods

This prospective observational study was conducted in outpatient clinic of Obstetrics and Gynecology Department of Al-Azhar University Hospital at New Damietta during the period from April 2016 to January 2017. An informed consent was taken from each woman participating in the study. The study included 50 women with polycystic ovary syndrome (PCOS) with the following criteria:

Inclusion criteria

Patients at childbearing period (18-40 years), patients diagnosed with polycystic ovary syndrome by Rotterdam criteria, if any 2 out of 3 criteria are present (oligoovulation or anovulation, hyperandrogenism and PCO by U/S: 12 or more 2-9mm ovarian follicles and/or ovarian volume exceeding 10 ml in one or two ovaries), Use of metformin alone for 3 months (850 mg/day) and Past history of infertility.

Exclusion criteria

Extremely aged <18, >40 years, other androgenic cause and Use of Clomiphene citrate or oral contraceptive pills. All eligible women in this study were subjected to: personal, menstrual history, other symptoms suggestive of PCOS: Infertility (sexual history, 1ry or 2ry, period of infertility, investigations done, treatment received), hirsutism and obesity, medical history including diabetes, hypertension.... etc. And drug history. Performing general examination including: Hair all over the body, the modified Ferriman Gallwey score is a qualitative tool for evaluating and quantifying hair growth in nine androgen-dependent areas in women. This scoring system evaluates nine different body parts (upper lip, chin, chest, upper back, lower back, upper abdomen, lower abdomen, arm, and thigh), with scores ranging from zero (no excessive terminal hair growth visible) to four (extensive hair growth visible) for each body part evaluated. A maximum score of 36 is possible, but a score of ≥ 8 typically indicates hirsutism (Brodell and Mercurio, 2010), a score of 8 to 15 indicates mild hirsutism, and a score greater than 15 indicates moderate or severe hirsutism. This scoring system has limitations because of the somewhat subjective nature of the assessment and the difficulty of evaluating women who have cosmetically removed their hair (Blume-Peytavi, 2013). Because increased androgen levels may also lead to pilosebaceous responses, such as acne, excessive sebum secretion, or diffuse or localized loss of hair, a dermatologic examination is mandatory (Blume-Peytavi, 2013). Also estimation of body weight, body height that was measured while standing with light clothing and no shoes and body mass index (BMI) was calculated as weight in kilograms (kg) divided by height in meter squared (m)² (<20: underweight, 20-25: optimal weight, >25: overweight, >30: obese, > 35: moderately obese, >40: morbidly obese). Transvaginal sonography, Laboratory investigations as: LH, FSH, fasting insulin, free testosterone and endometrial sample by pipette aspirator for androgen receptors. These investigations were done before and after three months of metformin treatment.

Sampling of blood

Three ml of venous blood were collected from each patient at 9.0 am in a sterile vacutainer tube, left to clot and then centrifuged at 3000 r.p.m., then serum was separated and stored at -20 till the time of hormonal assay.

Principles of Hormonal assay

All assayed hormones (FSH, LH, fasting insulin and free testosterone) were done using an automated chemiluminescence immunoassay by immulite instruments (Diagnostic products corporation, Los Angeles, CA 90045 USA).

Tissue preparation and immunohistochemistry

Endometrial pipelle samples were fixed in 10% buffered formaldehyde solution, embedded in paraffin blocks and cut into 5mm

sections. 2 slides were prepared from each specimen (one for hematoxylin and eosin (H&E) and the other slide for IHC). For immunohistochemical analysis, tissue sections were incubated in 60°C for 30 minutes, and then the sections were deparaffinized in xylene and rehydrated by alcohol. Antigen retrieval was performed by citrate solution in pH= 9 at 121°C for 20 minutes. To block the endogenous peroxidase activity, sections were immersed in 3% hydrogen peroxide for 30 minutes. Sections were incubated with anti-AR polyclonal antibody (dilution 1:500, Santa Cruz Biotechnology, CA) overnight at 4°C. After applying secondary antibody (biotinylated anti-rabbit immunoglobulin) associated with the Envision system, sections were washed in PBS and 3,3'-Diaminobenzidine tetrahydrochloride was used as chromogen and Mayer's hematoxylin as counterstaining. Immunopositive AR expression was restricted to nuclei of the endometrial glandular and stromal cells and appeared as brownish coloration. By omitting the primary antibody, sections were used as the negative control. Prostatic tissue was used as external positive control. Early proliferative phase was defined as days 4–7, Mid proliferative (days 8 - 10) and Late proliferative (days 11 - 14) without signs of secretory change. Histologic dating of secretory-phase biopsies was assigned using the criteria of Noyes et al (1950). The immunohistochemical evaluation was performed with the semiquantitative HSCORE ("histo" score) which is applicable to androgen receptors. The score was obtained by the formula: 3x percentage of strongly stained nuclei + 2x percentage of moderately stained nuclei + percentage of weakly stained nuclei, giving a range of 0 to 300 (Helena et al. 1996).

Statistical analysis of data

The collected data were organized, tabulated and statistically analyzed using statistical package for social sciences (SPSS) version 19 (SPSS Inc, Chicago, USA), running on IBM compatible computer. For qualitative data, frequency and percent distributions were calculated. For quantitative data, mean, standard deviation (SD), minimum and maximum were calculated. For comparison between two measurements, the independent samples (t) test was used. Pearson correlation co-efficient (r-test) was used for correlating different variables. For all tests p value <0.05 were considered significant. For all tests p value >0.05 were considered insignificant.

Results:

The results of the present study were presented in the following tables:

Table (1): Demographic data of studied cases.

Variable	Mean± SD
Age (years)	29.1 ± 5.7
BMI (kg/m ²)	26.5 ± 0.6
Parity	Range (0-2)

The mean age of the studied group was 29.1 ± 5.7 years, mean BMI was 26.5 ± 0.6 kg/m² and parity ranged from 0 to 2 (table 1).

Table (2): Comparison between studied cases before and after metformin administration as regard BMI.

Groups Parameter	Before	After intervention	P- value
BMI (kg/m ²)			
Mean± SD	26.5 ± 0.6	25.1 ± 0.39	<0.001

P value <0.001 indicate significance

There was a significant decrease of BMI of the studied cases after metformin administration.

Table (3): Comparison between studied cases before and after metformin administration as regard endocrine parameters.

Groups Parameter	Before	After intervention	P- value
FSH (mIU/ml)	4.78 ± 1.6	5.58 ± 2.76	> 0.05
LH (mIU/ml)	8.07 ± 2.48	6.85 ± 3.5	< 0.05*
Testosterone (pg/ml)			
	1.85 ± 0.96	1.83 ± 1.3	> 0.05
Fasting insulin (μIU/L)	13.5 ± 5.3	10.2 ± 6.9	< 0.05*

* P value < 0.05 significance

There were a significant decrease of LH level after metformin administration from 8.07 ± 2.48 mIU/ml to 6.85 ± 3.5 mIU/ml and a significant decrease of fasting insulin level from 13.5 ± 5.3 before metformin administration to 10.2 ± 6.9 after administration (table 3). There was a significant decrease of LH/FSH ratio in studied cases after metformin administration as compared with before administration (table 4).

Table (4): Comparison between studied cases before and after metformin administration as regard LH/FSH ratio.

Groups Parameter	Before	After intervention	T test	P- value
LH/FSH ratio	1.84 ± 0.14	0.95 ± 0.07	5.6	< 0.05*

Pathological findings

Before metformin treatment, the histopathologic examination of the endometrial specimens revealed features of early proliferative endometrium in 32 cases, late proliferative in 10 cases, while the remaining 8 cases showed features of simple endometrial hyperplasia. After treatment, 30 out of the 32 cases with early proliferative endometrium showed progression to late proliferative endometrium, while the remaining 2 cases showed no morphologic changes before or after treatment. The 10 cases diagnosed as late proliferative endometrium before treatment, progressed to early secretory (3 cases), mid-secretory (3 cases) and late secretory endometrium (4 cases) after treatment that indicate ovulation. As regard to IHC, we first compared endometrial AR expression in women with PCOS to that in normal fertile controls and the results revealed marked increase in AR expression in patients with PCO compared to normal fertile controls ($p < 0.001$). The HSCORE of AR in each cell type was compared in the proliferative and secretory phases and significant increase was observed in both epithelial and stromal AR in the endometrium of women with PCOS. (Epithelial: $p = 0.013$ for proliferative and $p < 0.0001$ for secretory phase), (stromal: $p = 0.005$ for proliferative and $p < 0.0001$ for secretory phase). In the present study, the highest androgen receptor expression was noted in cases with simple endometrial hyperplasia (*fig. 1*) (H-scores ranged from 210 to 260 - mean average 245), followed by cases diagnosed as early proliferative endometrium (*fig. 2*) (H-score range 130-180 with mean average 155) and lastly cases of late proliferative endometrium were ranged in H-score from 90 to 110 (mean average 98). Moderate decrease in AR expression was noted in cases progressed from early to late proliferative endometrium after treatment (30/32) (H-scores 110-130 with mean average 120). The two cases with no morphologic changes showed no significant difference in AR expression before or after treatment. As regard to the 10 cases progressed from late proliferative to secretory endometrium after treatment, marked decrease in AR expression was noted in both early (3/10) (H-score ranged from 10 to 20 with mean average 14) and mid-secretory cases (3/10) (H-score range 7-12 with mean average 9). AR immunonegativity was observed in late secretory (4/10) cases (H-score 0) (*fig. 3*). The overall staining, however, was reduced in each cell type during the secretory phase compared to the proliferative phase. In the early to midsecretory phase, epithelial AR immunostaining was markedly decreased along with reduced stromal AR expression. In the late secretory phase and after treatment, AR staining was depleted from the endometrial epithelium and stromal cells. By using the semiquantitative HSCORE assessment of immunohistochemical staining, both the stromal and glandular compartments of proliferative endometrium expressed greater AR than endometrium in the secretory phase of treated cases ($P = 0.005$).

Regression of hyperplasia was noted in 3 out of the 8 cases diagnosed as simple endometrial hyperplasia and showed marked decrease in AR expression (H-score 80-100 with mean average 85). The remaining 5 cases revealed only minimal to mild decrease in AR expression.

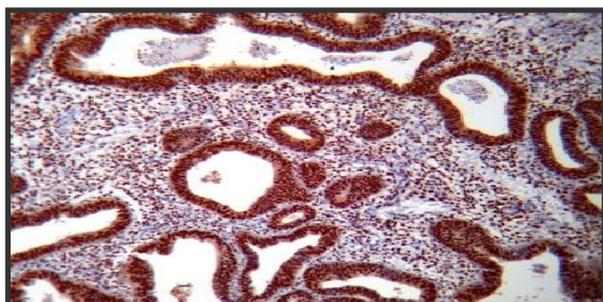


Fig. 1: Strong and diffuse immunoreactivity to AR in simple endometrial hyperplasia(x200)

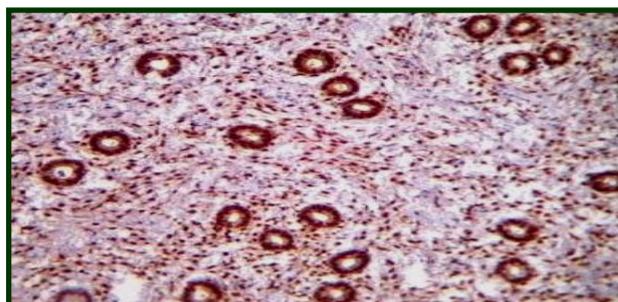


Fig. 2: Early proliferative endometrium showing diffuse and moderate epithelial and stromal immunoreactivity to AR (x100)

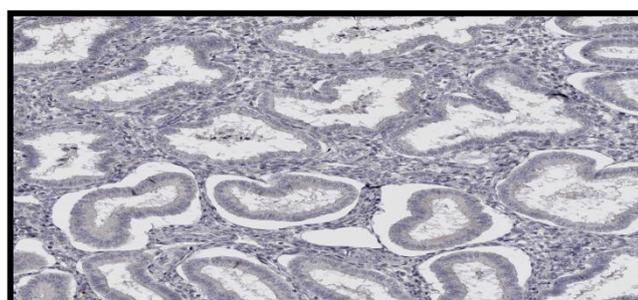


Fig. 3: Loss of AR immunoreactivity in late secretory endometrium (x200)

Discussion

The endocrine abnormalities associated with PCOS include dysregulation of the gonadotropin-releasing hormone (GnRH) pulse generator to feedback inhibition by ovarian steroids, resulting in luteinizing hormone (LH) hypersecretion and decreased follicle-stimulating hormone (FSH) and ovarian stromal–thecal hyperactivity, resulting in ovarian hyperandrogenism, all of which may lead to significant biochemical, reproductive and metabolic dysfunction (*Allahbadia and Merchant, 2011*). Insulin resistance is an important component in the etiopathogenesis of PCOS, being associated with obesity and hirsutism (*Rojas et al., 2014*). Metformin, a biguanide, increases insulin sensitivity in the liver to reduce gluconeogenesis and hyperinsulinemia. Clinical studies

have shown that administration of metformin to PCOS women resulted in decreased androgen levels and increased rates of spontaneous ovulation (*Kang and Paek, 2017*). In the present study, there were insignificant increase of FSH, significant decrease of LH level, insignificant decrease of free testosterone level and significant decrease of fasting insulin level after metformin administration and these agree with *Ganesan et al. (2011)* who reported that metformin reduces fasting insulin, testosterone and glucose levels. Metformin therapy to PCOS women lowered serum fasting insulin, total and free testosterone as well as estradiol levels at oocyte retrieval, enhanced clinical pregnancy and live birth rates, and diminished the risk of OHSS (*Kang and Paek, 2017*). In the present study, the cases who had higher fasting insulin, had menstrual irregularity more than who had lower fasting insulin. A retrospective study done by *Gonzalez et al. (2003)* reported that the higher degree of insulin resistance the greater prevalence of amenorrhea in women having polycystic ovary morphology. *Mellembakken et al. (2011)* found no difference in fasting insulin levels or insulin resistance between irregularly and regularly menstruating women with PCOS, although serum androgens were lower in the regularly menstruating group.

In the present study, 20 % of studied cases had regular menstrual cycle and ovulatory cycle after metformin administration. *Tosca et al. (2010)* studied the use of metformin 500 mg three times a day for 8 weeks in 22 women with PCOS who suffered chronic oligomenorrhea. They found that 21 women reported regular menstruation at the close of the study, with 86% of these women having ovulatory progesterone levels. *Kang and Paek (2017)* reported that 26% of patients with PCOS treated with metformin had the cumulative ovulation rate 75% (6/8) at 24 months follow up. Metformin was better than placebo for ovulation rate in overall women with PCOS (OR 2.12 [1.50,3.00] I²=69% p=0.000019; 13 studies, 875 participants), women with PCOS and a BMI ≤30kg/m² (OR 2.33 [1.43–3.81] I²=88% p=0.00071; 4 studies, 417 participants), women with PCOS and a BMI ≥30kg/m² (OR 1.94 [1.20–3.15] I²=39% p=0.0073; 9 studies, 458 participants) (*National Health and Medical Research Council, 2017*) and women with non-clomiphene citrate resistant PCOS (OR 3.55 [1.46–8.65]; 6 studies, 401 participants) (*Creanga et al., 2008*). However, there was significant statistical heterogeneity in overall women with PCOS and women with PCOS and a BMI ≤30kg/m². There was no difference in ovulation rate between metformin and placebo in women with clomiphene citrate resistant PCOS.

In the present study, the mean BMI was 26.5 ± 0.6 kg/m² which decreased after metformin administration to 25.1 ± 0.39 kg/m². *Aurelian and Anca (2017)* reported that metformin treatment administered in high doses (3000 mg/day) on a 6 months period was useful for weight loss as BMI levels decreased significantly in the first 3 months in metformin group (32.5±/-1.65 kg/m² vs. 28.4±/- 1.95 kg/m²), BMI variation in the non-metformin group was smaller and BMI levels had reached a plateau after 3 months of metformin treatment. *Afroz et al. (2017)* didn't agree as they found that in basal conditions, there was no difference between BMI in control group (M±SD; 27.14±3.89) & treated group (27.33±3.52). During the 3 months pharmacological treatment with metformin the mean BMI did not change in any group (control 26.97±3.91 vs treated 27.13±3.46, p= 0.848) (although there was a slight reduction in BMI of both groups). In the present study, 10 % of studied cases got pregnant after metformin administration. *National Health and Medical Research Council (2017)* reported that metformin was better than placebo for pregnancy rate in overall women with PCOS (OR 3.86 [2.18, 6.84] I²=0% p<0.00001; 6 studies, 479 participants) and women with PCOS and a BMI ≤30kg/m² (OR 4.41 [2.24–8.66] I²=40% p=0.000017; 3 studies, 250 participants) but no difference in women with PCOS and a BMI ≥30kg/m², women with clomiphene citrate naïve PCOS, women with clomiphene citrate resistant PCOS or women with non-clomiphene citrate resistant PCOS.

In the current study, we found that women with PCOS exhibited elevated endometrial AR expression compared to normal fertile controls and this increase was most apparent in glandular epithelium. Also, *Cloke and Christian. (2012)* reported that epithelial AR is up-regulated by estrogens and androgens and is inhibited by progestins, and, these results suggest that the poor reproductive performance observed in women with PCOS may be due, in part, to the concomitant increase in both serum androgens and elevations in endometrial AR. In the present study, we found higher expression of AR in proliferative than secretory phase and AR immunonegativity was observed in late secretory phase. These results were in agreement with results of *Villavicencio et al. (2006)*, who reported that there was more AR expression in the proliferative phases than in the secretory phases, and in the late secretory phase there was no immunostaining in any of the cell types. *Shang et al. (2012)* believed that androgens act as antagonists of estrogen and that high levels adversely affect priming of the endometrium in the follicular phase or development in the luteal phase. In addition to opposing the action of estrogen at its receptor, androgens could affect the endometrium directly through their own receptor, which has been shown to be expressed by the human endometrium.

In the current study, regression of endometrial hyperplasia was noted in 3 out of the 8 cases after treatment with metformin. *Costello and Eden (2003)* found that 62% of women with PCOS had regularization of their menses and metformin may reduce the endometrial hyperplasia. The overexpression of endometrial ARs in PCOS, as a result of estrogenic and androgenic stimulation, provides tangible evidence linking the abnormal hormonal milieu of PCOS with endometrial dysfunction (*Gregory et al., 2002*). In women with PCOS, endometrial stromal and epithelial cells show a 30% higher expression of estrogen receptor (ER) and similar expression of (AR), compared with normal women (*Maliqueo et al., 2003*). *Zhang and Liao (2010)* described that high androgen levels could cause insulin resistance in endometrial glandular epithelial cells, and metformin has the effect of reversing this insulin resistance caused by androgens. *Dumesic and Richards (2013)* found that androgens inhibit the expression of ERs and PRs, but in PCOS it is believed that the stimulatory effects of estrogen on ER and PR expression outweigh androgenic inhibition. In the present study, 10% of cases had gastrointestinal side effects of metformin which subsided after few days. Metformin had a higher incidence of gastrointestinal related adverse events compared to placebo (OR 9.23 [4.18, 20.37] I²=25% p<0.00001; 5 studies, 253 participants) in women with PCOS (*National Health and Medical Research Council, 2017*).

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