Reduced Steroid Synthesis in the Follicular Fluid of MTHFR 677TT Mutation Carriers: Effects of Increased Folic Acid Administration

Verminderte Steroidsynthese in der Follikelflüssigkeit von MTHFR-677TT-Mutations-Trägerinnen: Auswirkungen einer höheren Folsäuregabe

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ABSTRACT

Objective To compare steroid profiles in the follicular fluid (FF) from women homozygous for the methylenetetrahydrofolate reductase (MTHFR) 677C>T mutation and wildtype controls and to correlate it with the folic acid administration scheme applied at the time of oocyte retrieval.

Design Retrospective single center study.

Subjects and Methods Infertile patients treated by using assisted reproductive techniques were genotyped routinely for the MTHFR 677C>T mutation. In 2006 they had received folic acid supplementation doses of 400 µg daily per os. This group was designated Group-400 (n = 10). From 2008 onwards, all of our infertility patients received a daily dose of 800 µg folic acid per os. Women from this group were designated Group-800 (n = 28). FF were collected and a panel of steroid hormones (estradiol, estrone, estriol, cortisol, progesterone, 17-OH progesterone, testosterone, androstenedione, aldosterone, DHEA, and DHEA-S) was measured by isotope dilution liquid chromatography-tandem mass spectrometry employing atmospheric pressure photo ionization (APPI).

Results In Group-400, the FF hormone profile confirmed a significant reduction of estradiol in homozygous 677TT carriers (0.52 ± 0.08-fold, exact p = 0.032) and for the first time also revealed significantly reduced estriol concentrations in these individuals (0.54 ± 0.05-fold, p = 0.016), as compared to wildtype controls. In Group-800, no significant differences were found for concentrations of any of the steroid hormones between homozygous 677TT carriers and wildtype controls.

Conclusions The current findings support and extend previous reports on reduced concentrations of specific steroid hormones in follicular fluids of homozygous MTHFR 677C>T mutation carriers. The restoration of the FF hormone profile by elevated-dose folic acid supplementation might impact performing ART in infertile women with the MTHFR 677TT-genotype. Further adequately powered studies are necessary to verify our finding and to demonstrate the clinical effect of enhanced folic supplementation on ovarian function.

ZUSAMMENFASSUNG

Ziel Ziel dieser Studie war es, das Steroidprofil der Follikelflüssigkeit (FF) von Frauen mit Homozygotie für die Methylentetrahydrofolat-Reduktase-(MTHFR-)677C>T-Mutation mit dem Steroidprofil einer Wildtyp-Kontrollgruppe zu vergleichen und danach das Steroidprofil mit dem Folsäure-Dosierungsschema, welches zum Zeitpunkt der Eizellentnahme eingesetzt wurde, zu korrelieren.

Studiendesign Retrospektive monozentrische Studie.

Subjekte und Methoden Unfruchtbare Patientinnen, die sich einer assistierten Reproduktionstherapie unterzogen, erhielten routinemäßig eine Genotypisierung für die MTHFR-677C>T-Mutation. In 2006 wurden solche Patientinnen täglich mit 400 µg Folsäure oral supplementiert. Diese erste Gruppe wurde Gruppe-400 (n = 10) genannt. Ab 2008 erhielten alle unfruchtbare Patientinnen täglich eine orale Dosis von 800 µg Folsäure. Diese Gruppe wurde Gruppe-800 (n = 28) genannt. Bei beiden Gruppen wurden FF-Proben gesammelt und damit ein Hormonprofil der folgenden Steroidhormone (Östradiol, Östron, Östriol, Cortisol, Progesteron, 17-OH-Progesteron, Testosteron, Androstendion, Aldosteron, DHEA und DHEA-S) erstellt. Zur Messung wurde die Isotopenverdünnungs-Flüssigchromatografie gekoppelt mit Tandemmassenspektrometrie und atmosphärischer Photoionisation (APPI) eingesetzt.

Ergebnisse In der Gruppe-400 bestätigte das FF-Hormonprofil eine erhebliche Senkung des Östradiols bei homozygoten 677TT-Trägerinnen (0,52 ± 0,08-fach, exakt p = 0,032); es fanden sich auch zum ersten Mal erheblich verminderte Konzentrationen von Östriol bei diesen Frauen (0,54 ± 0,05-fach, p = 0,016) im Vergleich zur Wildtyp-Kontrollgruppe. In der Gruppe-800 gab es keine signifikanten Unterschiede bei den Konzentrationen der Steroidhormone zwischen homozygoten 677TT-Trägerinnen und der Wildtyp-Kontrollgruppe.

Schlussfolgerungen Die aktuellen Ergebnisse bestätigen und erweitern frühere Meldungen über verringerte Konzentrationen von bestimmten Steroidhormonen in der Follikelflüssigkeit von homozygoten MTHFR-677C>T-Mutations-Trägerinnen. Die Wiederherstellung des FF-Hormonprofils durch eine hochdosierte Supplementierung mit Folsäure könnte eine Auswirkung auf die Durchführung von ART bei unfruchtbaren Frauen mit dem MTHFR-677TT-Genotyp haben. Weitere adäquat gepowerte Studien werden benötigt, um unsere Ergebnisse zu bestätigen und die klinischen Auswirkungen einer höheren Supplementierung mit Folsäure auf die ovarielle Funktion zu zeigen.

Introduction

The one-carbon metabolism is an essential biological process that regulates the biosynthesis of nucleosides and the methylation of proteins, lipids, and DNA. Epidemiological evidence suggests that genetic variants of enzymes in the one-carbon metabolism pathway as well as folate intake influence ovarian function [1, 2, 3]. The enzyme 5,10-methylenetetrahydrofolate reductase (MTHFR) plays a key role in one-carbon metabolism as it catalyzes the reduction of 5,10-methylenetetrahydrofolate to 5-methyltetrahydrofolate, a methyl donor for the remethylation of homocysteine to methionine.

The common 677C>T mutation of the MTHFR gene, which results in an alanine-to-valine substitution at amino acid position 222, leads to a thermolabile variant of the enzyme with 70 % reduced activity [4, 5]. The prevalence of homozygous 677TT and heterozygous 677CT carriers is about 4–12% and 40–50% in the Caucasian population respectively [6]. The 677TT genotype is associated with a mild to moderately elevated serum homocysteine concentration, particularly in patients with insufficient folate supply [7, 8, 9, 10].

MTHFR gene variants have been correlated with the increased risk of neural tube [11, 12] and congenital heart defects [13, 14] as well as recurrent pregnancy losses [3, 15, 16]. Interestingly, folic acid supplementation has been reported as being potentially effective in reducing the risk of such conditions [17, 18, 19, 20].

We have previously demonstrated that homozygous 677TT carriers, when undergoing controlled ovarian hyperstimulation and ovulation induction protocols, show a reduced ovarian responsiveness as they require more r-FSH during ovarian hyperstimulation, produce significantly fewer oocytes, and have lower maximal serum estradiol (E2) concentrations [21]. Moreover, we have reported significantly reduced E2 in follicular fluid and reduced E2 synthesis in granulosa cells (GC) of homozygous 677TT individuals compared with heterozygous 677CT and homozygous 677CC carriers [22].

In contrast to our previous study [22] Rosen et al. reported that in his US-American study group, the C677T mutation was not associated with diminished response to ovarian stimulation. According to Rosen this could be due to a higher intake of folic acid in addition to the recommended 400 µg of folic acid per day. This appears likely because foods such as breads and cereals in the United States are fortified with folic acid.

The exact mechanism of folic acid action has not been entirely clarified. It has been shown, that folic acid supplementation can reduce elevated concentrations of homocysteine and compensate for effects of the MTHFR 677C<T mutation [24]. We propose that increased folic acid supplementation might also reverse the hor-

monal changes previously described by our group [21, 22]. These studies had been conducted in patients receiving a folic acid supplementation of 400 µg daily which was recommended at that time in order to reduce the risk of neural tube defects [25]. By the time the current study was designed, a daily administration of at least 800 µg of folic acid was proposed to be effective in NTD prevention [26, 27]. Thus, we altered our clinical practice accordingly. Since then, all of our patients receive a daily folic acid supplementation of 800 µg. The prospective investigation of the effect of a daily folic acid administration of 400 µg vs. 800 µg on homozygous MTHFR 677TT would require a recruitment of women supplemented with low folic acid daily doses. However, according to the present guidelines, a low folic acid supplementation would be considered unethical. Therefore, we tested our hypothesis in a retrospective analysis of DNA and follicular fluid probes stored from infertility patients who had received 400 µg or 800 µg folic acid daily while undergoing oocyte retrieval after controlled ovarian stimulation (COH) in our institution in 2006 and after 2008. This retrospective case control study forms the basis of this report.

Subjects and Methods

Patients

Due to internal protocols, infertility patients treated in 2006 had received daily folic acid supplementation doses of 400 µg. This group was designated Group-400 and we chose 10 infertile patients from the Centre for Reproduction, Munich, that met the inclusion criteria (below) and had stored FF available. From 2008 onward, all patients took 800 µg folic acid daily per os. This group was designated Group-800. Exclusion criteria involved

1. signs or symptoms of anomalies such as uterine fusion defects or submucosal fibroids, and acute inflammation,
2. an abnormal hormone profile (values for cycle day 3 FSH, LH, estradiol, testosterone, DHEA-S, prolactin, SHBG, and TSH), and
3. hepatitis B/C and HIV infections.

In all patients, protective titers against rubella virus were also confirmed. Individuals in Group-400 and Group-800 were matched according to their ovulation induction management and efficacy. As a result, we selected infertile Group-800 patients from the same center with frozen FF available, that matched age, total dose of FSH, days of stimulation and number of oocytes retrieved (n = 28). Additionally, both groups were selected, so that half of the patients per group was wildtype 677CC and the other half was homozygous 677TT and no significant difference were present between wildtype 677CC and homozygous 677TT carriers (▶ **Table 1**).

MTHFR 677C>T genotyping and the use of follicular fluids was approved by the Ethics Committee of the University of Munich (LMU), and written informed consent was obtained from all participants. The study was carried out according to the guidelines of the Declaration of Helsinki (clinical trial registration number: 178/0).

Folic acid supplementation

During 2006, all women were supplemented with 400 µg folic acid per os daily according to the existing recommendations [25, 28, 29]. Treatment was initiated at least one month prior to any ART procedure and continued at least up to pregnancy detection. Due to the recommendations [26, 27] of 2008, the daily folic acid dose was increased to 800 µg. Thus, the current study had two arms: Group-400 refers to women supplemented with 400 µg folic acid/day, while Group-800 refers to women receiving 800 µg daily.

▶ **Table 1** Main clinical features of the patients included in this study. Patients were matched regarding their ovulation induction management and efficacy. As a result, no significant difference was noted between the wildtype 677CC and the homozygous 677TT carriers.

	MTHR 677C>T mutation status		
	Wildtype CC	**Homozygous TT**	**p**
Group-400 (n = 10)			
Age	34.80 ± 1.39	36.40 ± 1.20	NS
BMI	24.20 ± 2.03	27.80 ± 2.17	NS
Total FSH (IU)	1900.00 ± 269.72	2210.00 ± 152.80	NS
Days of stimulation	10.20 ± 1.59	10.80 ± 1.62	NS
Oocytes retrieved	14.40 ± 2.71	12.40 ± 2.71	NS
Group-800 (n = 28)			
Age	36.21 ± 0.94	35.143 ± 0.94	NS
BMI	22.15 ± 0.55	21.95 ± 0.64	NS
Total FSH administered	1779.64 ± 100.86	2021.429 ± 89.86	NS
Days of stimulation	10.14 ± 0.32	10.42 ± 0.30	NS
Oocytes retrieved	14.57 ± 1.42	11.85 ± 1.18	NS

NS: not significant

Treatment protocol

The "long" protocol was used, as described elsewhere [30]. Starting between day 20 and 23 of the pre-treatment cycle, patients received the gonadotrophin-releasing hormone agonist nafarelin (Synarela, Pharmacia GmbH, Karlsruhe, Germany) by twice-daily nasal spray applications for at least 10 days. When pituitary down-regulation was confirmed (LH < 5 mIU/ml, E2 < 50 pg/ml) and double endometrial width was less than 5 mm, ovarian stimulation was started, using recombinant (r-) FSH (Puregon; Essex Pharma, Munich, Germany) with a daily subcutaneous dose of 150–200 IU. FSH doses were maintained or adjusted according to transvaginal sonographic folliculometry at stimulation day 8. When > 3 follicles reached a mean diameter of at least 17 mm, 250 µg of recombinant chorionic gonadotrophin (Ovitrelle, Serono, Unterschleißheim, Germany) were administered subcutaneously. Oocyte retrieval was performed under general anaesthesia by transvaginal, ultrasound-guided aspiration 36 hours later by a single person (U.N.) blinded to the MTHFR 677C>T status.

DNA isolation and analysis of the MTHFR 677C>T mutation

All of our infertility patients were routinely genotyped for the MTHFR 677C>T mutation. EDTA blood was drawn und genomic DNA was extracted from leukocytes with the QIAmp DNA blood mini kit (QIAGEN, Hilden, Germany), according to the manufacturer's protocol. Samples were genotyped by employing the 5' nuclease assay for allelic discrimination on an ABI PRISM 7700 sequence detection system (Applied Biosystems, Foster City, CA). The fluorogenic, allele-specific oligonucleotide probes and PCR primers were provided by Applied Biosystems (TaqMan genotyping assay ID: C_1 202 883_20). PCR was performed in 96-well plates in a total volume of 10 µL containing 5 µL TaqMan Universal PCR Master Mix (Applied Biosystems), 0.5 µL primer/TaqMan probe mix, and 10 ng genomic DNA. The PCR profile included an initial denaturation step at 95 °C for 10 min and 40 cycles at 92 °C for 15 s and 60 °C for 1 min. For allelic discrimination, a post-amplification evaluation was done using the ABI Sequence Detection Software supplied with the instrument.

Follicular fluid collection, storage, and analysis

After transvaginal aspiration, all retrieved oocytes were removed and denuded immediately for the subsequent ICSI procedure. FF from all follicles of the same patient were pooled and centrifuged immediately at 1000 xg for 10 min. Supernatants were aliquoted and stored at − 70 °C until further analysis. Samples were retrieved, thawed, and used for further analysis, after individual groups (i.e. 400 versus 800) and subgroups (i.e. MTHFR 677CC versus 677TT) had been designated according to the criteria described above.

Determination of the folic acid concentration in the follicular fluid

The folic acid content in the FF of Group-400 and Group-800 individuals was compared by off label use of a competitive chemiluminescent enzyme immunoassay kit on an automated Immulite 2000 immunoassay system. According to the manufacturer's (DPC, USA) protocol, this assay is intended for quantitation of folic acid in human serum and plasma.

Determination of steroid hormones by isotope dilution liquid chromatography-photospray ionization tandem mass spectrometry

Aliquots of frozen FF were shipped overnight on dry ice to the Georgetown University Medical Center (GUMC) Bioanalytical Mass Spectrometry Core Laboratory, Washington, DC. The research staff of the GUMC was blinded to the MTHFR 677C>T genotype. Isotope Dilution Liquid chromatography-tandem mass spectrometry (LC-MS/MS) was used in order to simultaneously measure the following steroid hormones: 17β estradiol, estrone and estriol in the estrogen profile method [31] and, cortisol, progesterone, 17-OH progesterone, testosterone, androstenedione, aldosterone, DHEA, and DHEA-S in the state of the art steroid profile method [32, 33].

An API-5000 triple-quadrupole mass spectrometer (Sciex, Concord, Canada) coupled with a photospray source and a HPLC system (Shimadzu Scientific Instruments, Columbia, MD) was used for the determination of the steroid profiles, employing isotope dilution with a deuterium-labelled internal standard (IS) for each analyte. Estrogen measurements were performed with the same instrument using an ESI ionization source.

300 µl of IS solution diluted in acetonitrile was added to a serum sample volume of 200 µl for deproteinization. After centrifugation at 13 000 rpm for 10 min, 450 µl of the supernatant was mixed with 900 µl of water, and a 1000-µl aliquot was transferred into the LC-APPI-MS/MS system onto a C8 column. After 3 min of washing with 3 % methanol in ammonium acetate followed by a steroid elution with a water/methanol gradient, the sample was injected into the mass spectrometer which continuously recorded two MRMs, one for each analyte and one for each deuterated internal standard corresponding to the labeled and the non-labeled hormones.

Statistical analysis

We performed an observational study using matched controls. Results are expressed as mean ± standard error of the mean (SE). Statistical significance was assessed by using the nonparametric Mann-Whitney U test. Analysis of the data was performed with the Statistical Program for Social Sciences 18.0.1 (SPSS Inc., Chicago, IL). Significance was assessed by two-sided exact p and was considered to be reached at an exact P value < 0.05.

Results

Effects of increased folic acid dosage on the folic acid concentration in the follicular fluids

Folic acid contents were compared in the FF of Group-400 and -800 and this confirmed significantly increased folic acid contents in the FF of Group-800 versus Group-400 patients (23.44 ± 1.38 vs. 11.91 ± 1.62 ng/ml respectively, exact $p < 10^{-3}$). This difference was confirmed also, when Group-800 and Group-400 patients carrying the 677CC genotype (24.06 ± 1.54 vs. 13.63 ± 1.86 ng/ml respectively, exact p = 0.001) and the 677TT genotype

(22.82 ± 2.34 vs. 10.20 ± 2.62 ng/ml respectively, exact $p = 0.005$) were compared (▶ **Fig. 1**).

Effect of increased folic acid administration on FF steroid hormones in 677TT and 677CC carriers

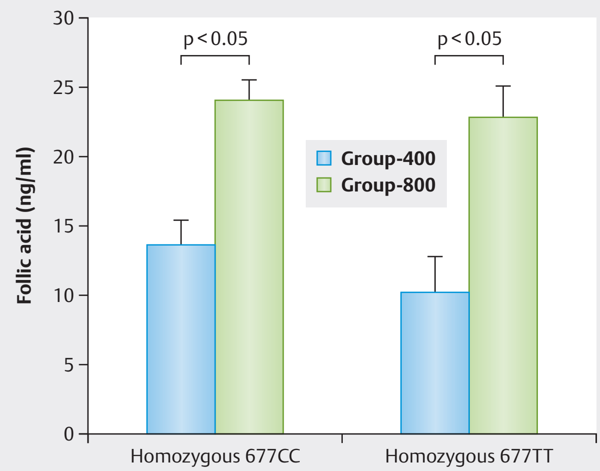
In Group-400, FF concentrations of E2 were significantly lower in 677TT than in 677CC individuals (0.52 ± 0.08-fold, exact $p = 0.032$). In addition, estriol (E3) concentrations also were significantly lower in 677TT individuals (0.54 ± 0.05-fold, $p = 0.016$). A marginally non-significant reduction was noted in 17-OH progesterone concentrations of 677TT carriers (0.78 ± 0.06-fold, exact $p = 0.056$) and no differences were observed between 677TT and 677CC individuals of the Group-400 for FF concentrations of the other measured steroids (▶ **Fig. 2**).

Within Group-800, none of the analyzed steroid hormones showed significantly different concentrations in follicular fluids of 677TT and 677CC individuals (▶ **Fig. 2**).

Discussion

We previously reported significantly reduced E2 in serum [21], FF, and GC cultures [22] of infertile homozygous 677TT individuals when compared with 677CC homozygotes. These data suggested an influence of the MTHFR 677C>T mutation on ovarian steroidogenesis. Increased folic acid supplementation having been shown to compensate for MTHFR 677T-effects [17, 18, 19, 20], we previously suggested this intervention might also restore ovarian steroidogenesis [21, 22]. Since 2008, all our infertile patients undergoing assisted reproductive treatments received a higher dose of folic acid and this prompted this retrospective comparison of steroid metabolite concentrations in follicular fluids of patients receiving different doses of folic acid supplementation. This approach was limited to infertile IVF/ICSI-patients where we had frozen FF samples available, resulting in a small number of selected patients. Retrospective design is an additional limitation of this study. While previous studies had been done by using immunometric E2 measurement, this study employed liquid chromatography-tandem mass spectrometry (LC-MS/MS), allowing more specific and sensitive analyses of a wider spectrum of steroids. Compliance with our recommendation of supplementing 400 µg or 800 µg of folic acid daily in the 400- or 800-group respectively was supported by significantly higher contents of folic acid in FF of the 800-group individuals (▶ **Fig. 1**). In women supplemented with 400 µg folic acid daily, we confirmed significantly reduced E2 concentrations in FF of homozygous 677T allele carriers. In addition, our mass spectrometric analyses demonstrated for the first time that 677TT homozygotes also had reduced concentrations of E3 when compared to 677CC individuals. This is intriguing, as intrafollicular E3 has been shown not to correlate with E2-concentrations, nor with size or maturity of ovarian follicles. Indeed, intrafollicular E3 is not believed to be an end-product of E2 metabolism and rather the result of an autonomous pathway of production from granulosa and theca interna cells [23]. Our report for the first time suggests that such pathways also may be affected by the MTHFR 677TT-genotype in women with inadequate folate supply.

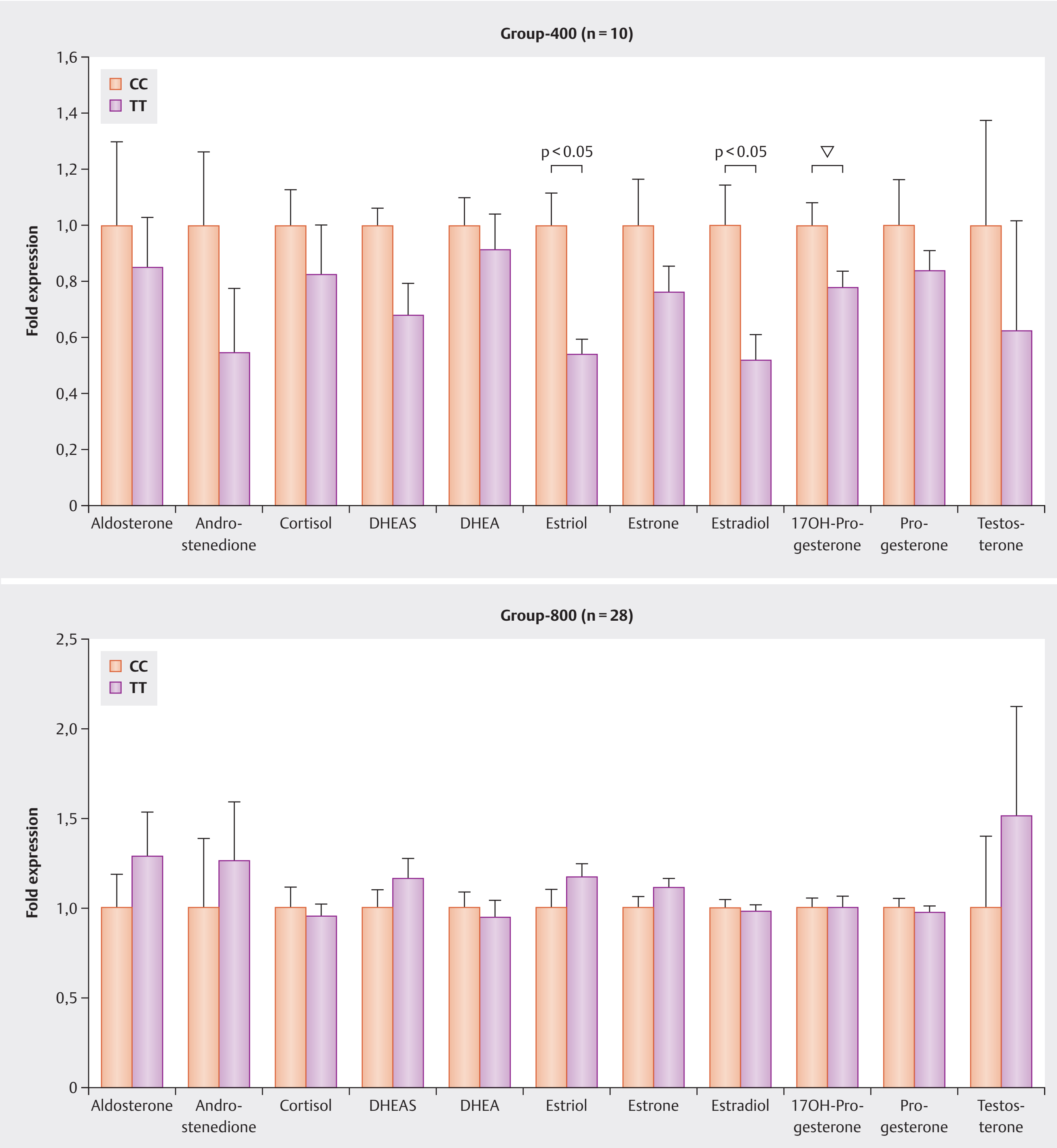
Steroid hormones are derivates of cholesterol, which is converted into pregnenolone, a precursor for the production of all consecutive steroid hormones. The biosynthesis of steroids follows several pathways and therefore requires a battery of enzymes, mainly dehydrogenases and cytochrome P450 oxygenases. Although our sample size was rather small and the study was retrospective and limited to infertile women, our results suggest that the MTHFR 677C>T mutation may be associated with affected activities of enzymes involved in the steroid metabolism, especially CYP19 (aromatase), as CYP19-dependent steroids were predominantly reduced in 677TT carriers. We also observed a marginally non-significant reduction of 17-OH progesterone. It could thus be speculated that the MTHFR 677C>T mutation also is associated with an impaired CYP17 (17α-hydroxylase/17,20-lyase)-catalyzed conversion of progesterone into 17-OH progesterone. Since this enzyme is present only in theca cells, the MTHFR 677C>T effect might influence also the metabolism of theca cells, in addition to GC.



▶ **Fig. 1** Folic acid concentration in the follicular fluid of both homozygous 677TT carriers and wildtype 677CC controls. A significant increase in folic acid concentration was achieved when administering 800 µg folic acid per os daily (Group-800, n = 28) compared to the corresponding 400 µg folic acid per os daily supplementation (Group-400, n = 10).

The biological background of our observations is not known. MTHFR catalyzes the conversion of 5,10-methylenetetrahydrofolate (5,10-MTHF) into 5-methyltetrahydrofolate (5-MTHF). 5-MTHF itself, the biologically most active form of folate, functions as a methyl group donor for the remethylation of homocysteine to methionine. This is an important step in the metabolic network that regulates the biosynthesis of nucleosides and the methylation of proteins, lipids, and DNA.

On the level of ovarian cell function, the MTHFR 677C>T mutation may interfere with the complex regulation of steroid biosynthesis and metabolism. To completely understand the underlying mechanisms, it may be necessary to study genes, which are involved in the process of ovarian steroidogenesis and whose methylation may be altered by the MTHFR 677C>T mutation.



▶ **Fig. 2** Hormone profile of the follicular fluid of homozygous 677TT carriers and wildtype 677CC controls under the effect of a daily dose of 400 µg (Group-400) or 800 µg (Group-800) folic acid. A significant reduction in estradiol and estriol concentrations was observed in carriers of the homozygous 677TT genotype that received 400 µg folic acid (Group-400). We also observed a marginal non significant reduction of 17-OH progesterone concentrations in these samples (triangle). No MTHFR-dependent differences were found in patients receiving daily doses of 800 µg folic acid (Group-800).

Apart from alterations in DNA methylation patterns, the elevated plasma homocysteine observed in homozygous T/T subjects might have a direct influence on ovarian steroidogenesis. Its role in inducing oxidative damage and stress has been well documented in many studies. Homocysteine contains a reactive sulfhydryl group, promoting oxidation reactions and the formation of reactive oxygen species [34] which can result in a significant damage of proteins and the cell structure. Moreover, there is growing evidence that homocysteine leads to changes in the concentrations of other plasma aminothiols, generating a pro-oxidant milieu by lowering the concentration of reduced cysteine [35]. In this way, hyperhomocysteinemia might directly affect or modify particular proteins and/or enzymes involved in ovarian steroid hormone biosynthesis and thus also lead to reduced steroid concentrations in homozygous 677TT individuals.

Interestingly, the FF hormone profile of 677TT carriers is restored to the normal values of homozygous 677CC carriers by administering an increased dosage of 800 instead of 400 µg folic acid. Such an observation could be of clinical importance.

In fact, some recent clinical studies could demonstrate an association between higher folate intake with increased fecundability [36], lower risk of anovulation [37] and ovulatory [1] infertility. In addition, Kadir et al. in a recent study reported significantly increased antral follicle counts in women with 0,8 mg of folic acid daily [38].

Taking into account that homozygous 677TT carriers are expected to present with low ovarian responsiveness and thus reduced oocyte output, sufficiently high folic acid intake appears to improve ART outcome. Indeed, results of a prospective cohort study suggest increased higher folate intake was related to a higher probability of live birth among women undergoing assisted reproductive technology [39].

In conclusion, our findings support previous studies suggesting that folate metabolism influences synthesis of E2, and potentially also other ovarian steroids, such as estriol and 17-OH-progesterone. Our observations could help to gain further insights into the mechanisms related to the MTHFR 677C>T mutation and its effects on folliculogenesis and ovarian steroid synthesis.

Capsule

Increased folic acid administration results in the restoration of the follicle fluid hormone profile which may have an impact on the success of controlled ovarian stimulation in infertile women presenting with reduced MTHFR activity.

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Conflict of Interest

The authors declare that they have no conflict of interest.

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