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Evaluating the Effect of Epigallocatechin Gallate (EGCG) in Reducing Folate Levels in Reproductive Aged Women by MTHFR and DHFR Genotype in Combination With Letrozole or Clomiphene

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ABSTRACT

Previous epidemiological studies have suggested that green tea catechins, including Epigallocatechin-3-gallate (EGCG), the most abundant polyphenol in green tea, may be associated with reduced serum folate levels. This is of particular interest as women of childbearing age may be consuming EGCG from tea, dietary supplements, or involved in active clinical trials studying EGCG or green tea extract. EGCG was reported to shrink uterine fibroids in preclinical and clinical studies. This observation led to the development of a multicenter NICHD-funded clinical trial to evaluate the safety of EGCG for treating women with fibroids and unexplained infertility (NCT04177693). To answer the question of whether green tea extract standardized to EGCG led to a reduction in folate, 39 women aged ≥18 to ≤40 years, with/without uterine fibroids, were evaluated. These women were randomized to receive either EGCG, EGCG + clomiphene, or EGCG + letrozole for 30 days. A daily dose of 720 mg of highly characterized green tea extract containing EGCG was used. Participants were genotyped for polymorphisms at positions 677 and 1298 in MTHFR and for the −19 bp deletion polymorphism of DHFR. During the intervention with EGCG, folate levels remained in the normal range in all subjects. Our data suggest that in reproductive-age women, a 30-day course of EGCG 720 mg daily taken alone or in combination with clomiphene citrate or letrozole (for 5 days) is well-tolerated and is not associated with folate deficiency even in the presence of MTHFR and/or DHFR polymorphisms known to negatively impact folate synthesis.

Trial Registration: Clinical trial: NCT 01311869

Summary

- What is the current knowledge on the topic?
  - At present, mechanistic evidence for EGCG inhibiting folate synthesis consists of molecular modeling and cell-free mechanistic studies.
- What question did this study address?
  - This study was a randomized phase IB clinical trial with patients receiving EGCG for at least 30 days, with folate levels measured before and following administration of the study agent EGCG.
- What does this study add to our knowledge?
  - To our knowledge, this is the first study that evaluated EGCG in a controlled clinical trial, which found that EGCG treatment for 1 month in healthy reproductive-age women, regardless of MTHFR and DHFR genotype, did not result in folate deficiency.
- How might this change clinical pharmacology or translational science?
  - A longer clinical trial involving EGCG administration in reproductive-age women (i.e., FRIEND NCT05364008) is ongoing and will continue to assess folate levels in women receiving EGCG to address any potential safety concerns.

1 | Introduction

Green tea (*Camelia sinensis*), next to water, is the most consumed beverage in the world, containing polyphenols that include epicatechin, epicatechin gallate, epigallocatechin gallate (EGCG), and alkaloids [1]. The inhibitory effects of EGCG on uterine fibroids may have an important potential clinical application. Al-Hendy et al. reported that EGCG inhibits catechol-o-methyltransferase (COMT), decreases transforming growth factor-β3 production, and upregulates the gene expression of bone morphogenic protein-2 [2–4]. These effects inhibited fibroid tumor formation in vivo in nude mice [5]. The EGCG-induced antifibroid preclinical findings in fibroid animal models and human fibroid cell lines were translated into an NIH-funded double-blinded, placebo-controlled randomized clinical trial. We have reported on the liver safety of EGCG in women of childbearing age [6]. Moreover, that study showed significant reductions in total uterine fibroid volume and fibroid-specific symptom severity in subjects randomized to receive green tea extract compared with placebo, along with a favorable green tea extract safety profile. The United States Pharmacopeia identified 51 case reports of hepatotoxicity associated with green tea extract using daily doses ranging from 500 to 3000 mg daily, equivalent to 250 to 1800 mg of EGCG daily [7]. The USP recommendations did state that green tea extract should be taken with food to avoid hepatotoxicity. In addition, the European Food Safety Authority panel concluded that intakes above 800 mg of EGCG daily significantly increased serum transaminase levels compared with control [8]. In a recent multi-site study, the hepatic safety profile of EGCG was assessed in reproductive-aged women receiving EGCG alone, EGCG and clomiphene, or EGCG and letrozole, and no subjects demonstrated any signs of drug-induced liver injury [6].

Dihydrofolate reductase (DHFR) and methylenetetrahydrofolate reductase (MTHFR) are two critical enzymes that regulate folate synthesis by converting folic acid to its active form, L-methylfolate, which is critical for DNA [9, 10]. Polymorphisms in MTHFR and an insertion/deletion of DHFR can also further reduce the conversion of folic acid to the active folate form. DHFR is a key enzyme in the folate pathway that converts folate to dihydrofolate (Figure 1) and is a primary target for inhibition by "antifolate" drugs, including methotrexate (MTX) and trimethoprim (TMP). EGCG has been studied extensively for its anti-cancer properties, with one proposed mechanism of EGCG suggested to inhibit DHFR, leading to anti-cancer activity [11].

A DHFR insertion/deletion polymorphism is highly prevalent, with the frequency of the del/del genotype reported between 10.5% and 48% in various populations [12–16]. The prevalence of MTHFR polymorphisms can range from 30% to 70% with wide variation across different patient populations [17–19].

1.1 | Objective

The primary objective of this analysis was to determine if green tea extract standardized to EGCG increases the risk of folate deficiency in women. A secondary analysis was to determine if green tea extract standardized to EGCG in combination with DHFR/MTHFR polymorphisms further increases the risk of folate deficiency. To our knowledge, this is the first controlled clinical trial to assess if green tea extract standardized to EGCG reduces folate levels in women of childbearing age who were genotyped for DHFR and MTHFR polymorphisms.

2 | Materials and Methods

2.1 | Reagents

QIAamp blood Mini Kit and Hot Star Taq master mix were purchased from QIAGEN (Maryland, USA). DNase/RNase-free water was purchased from Zymo research (Irvine, California).

2.2 | Clinical Trial Design

The design of this study and evaluation for hepatic safety has been previously reported [6]. Epigallocatechin Gallate (EGCG) For Treatment Of Unexplained Infertility Associated With Uterine Fibroids (Pre-Friend Trial): Early Safety Assessment (NCT04177693). Women were randomized to three arms that included (1) Green Tea Extract standardized to 45% EGCG (GTE), (2) GTE and clomiphene citrate, or (3) GTE and letrozole. GTE was initiated before the menstrual cycle. For subjects randomized to the oral fertility medication arms, clomiphene citrate or letrozole was initiated between Days 2 and 5 of the menstrual cycle and taken for 5 days. Five visits were conducted throughout the study that included an initial screening visit followed by four study visits during treatment as presented in Figure 2. A blood sample was obtained to measure folic acid during the screening visit and during the last study visit. Folic acid was measured using a validated electrochemiluminescent

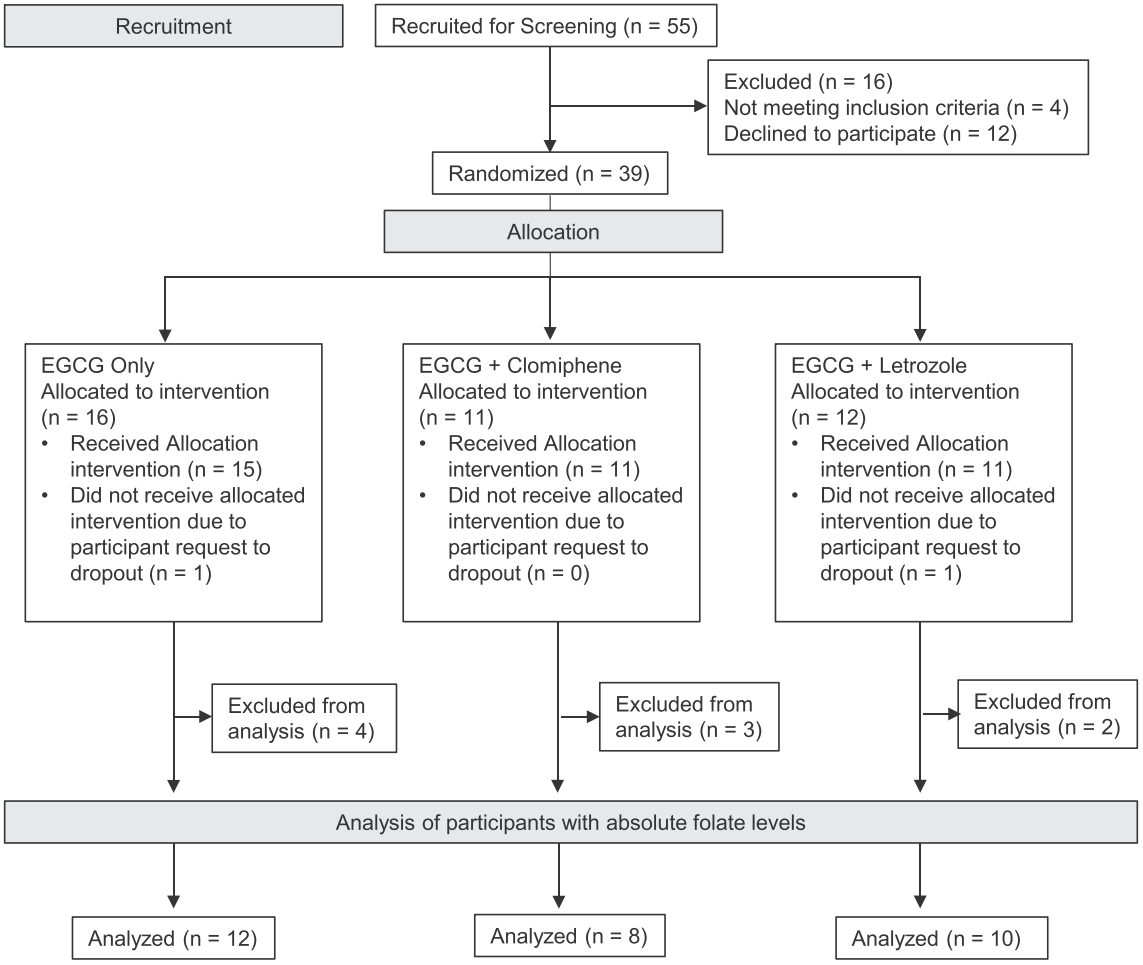

FIGURE 1 | Flow chart of study population; EGCG: epigallocatechin gallate.

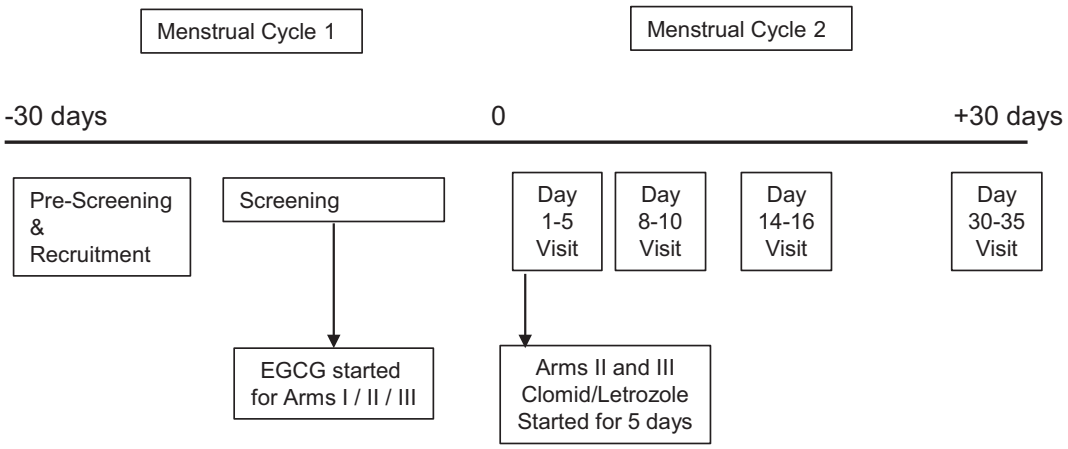

FIGURE 2 | Visit timeline. Treatment Arm I is the green tea extract (EGCG) only for the duration of the study, Arm II is the green tea extract (EGCG) and 5 days of clomiphene citrate, and Arm III is the green tea extract (EGCG) and 5 days of letrozole. Day 1 is the start of the subject's first menstrual cycle after the screening visit. EGCG: epigallocatechin gallate.

assay manufactured by Roche at each respective clinical site. The study protocol was reviewed and approved by a single institutional review board (IRB, John Hopkins University) as required by the National Institutes of Health, with subsequent acknowledgment by the local IRB at each participating clinical site as well as the Data Safety Monitoring Committee. All participants provided informed written consent in accordance with the single and local IRBs. Clinical sites included The University of Chicago, University of Illinois at Chicago, Johns Hopkins University, and Yale University.

2.3 | Study Agents

Green Tea extract was standardized to 45% EGCG under IND (#150951) and was supplied by Beehive Botanicals Inc. (Hayward, Wisconsin). Each capsule of GTE contained 400 mg of green tea extract. Subjects were instructed to take four capsules of GTE daily with the breakfast meal. The daily dose of four capsules of GTE provided a daily EGCG dose of 720 mg. Quantity of the four catechins was determined by LC-MS/MS. Clomiphene citrate (100 mg) or letrozole (2.5 mg) was administered for 5 days.

2.4 | Isolation of Genomic-DNA

Whole blood samples were collected from a peripheral vein in all subjects and stored at −80°C until further processing. DNA was isolated from the blood samples using the QIAamp blood Mini Kit. The 200 μL of the blood sample was mixed with 20 μL of the protease. DNA was purified as described in the protocol of the kit. The yield of the isolated DNA was determined with a BioTek CYTATION 5 plate and imaging reader (Vermont, USA). Quantification was performed by spectrophotometry at 260 nm. Isolated DNA samples were stored at −80°C until use.

2.5 | Polymerase Chain Reaction

DNA isolated from the blood samples was used as a template DNA for the PCR amplification of MTHFR 677, MTHFR 1298, and DHFR 19 bp. The forward and reverse primers for the target genes were designed (Table 1) and synthesized from Integrated DNA Technologies (IDT). The PCR amplification cycle of all three genes was optimized before running the PCR cycle in an Eppendorf Mastercycler (Hamburg, Germany). Each PCR reaction contained 1 μL each (10 μM) forward and reverse primers, 50 ng template DNA, and 15 μL Hot star Taq master mix. The total volume of the reaction was made up to 30 μL with nuclease-free water. The mixture was mixed well and centrifuged at 500 g for 5 min before running the PCR cycle. The PCR cycle for MTHFR 677 and DHFR started with denaturation at 95°C for 15 min followed by denaturation, annealing, and extension at 95°C for 30 s, 62°C for 30 s, and 72°C for 45 s, respectively. Similarly, MTHFR was denatured at 95°C for 15 min followed by denaturation, annealing, and extension at 95°C for 45 s, 62°C for 45 s, and 72°C for 1 min, respectively. These steps were repeated 40 times for all three genes. The PCR reactions were cleaned up and 2 μL of them was placed on a BioTek TAKE3 micro-volume plate (Vermont, USA) to determine the concentration. The concentration of the PCR products was determined with a BioTek CYTATION 5 plate and imaging reader (Vermont, USA). The PCR template concentration was maintained at 10–20 ng/μL before being submitted to Sanger sequencing.

2.6 | Statistical Analysis

Data are expressed as mean ± SD or median (interquartile range) for the continuous variables. Student's *t* test was used for testing differences between two groups. Analysis of variance test was used for testing differences among the three groups. Categorical data are reported as frequencies and percentages. Differences in these measures between treatment groups were assessed by chi-squared analysis. Fisher's exact test was used for expected frequencies of < 5. Analysis was done using the SAS version 9.4 software. Significance was defined as a two-sided $p < 0.05$.

3 | Results

3.1 | Demographics of Study Participants

This was a Phase 1B clinical trial involving non-pregnant, non-lactating women aged 18 to 40 years old, whether or not they had uterine fibroids. Of the 39 participants that were randomized, 30 completed all five study visits, and 30 of them had folate levels determined during screening and at the end of the study. No drop-out was attributed to any adverse effects from study agents. Figure 1 shows the flowchart of the study population. Baseline demographics and participant information are presented in Table 1. Participants had a mean age of 29 years and a mean body mass index of 26.9 kg/m^2. These characteristics were similar among the three treatment groups. In addition, the mean baseline AST (20.9 U/L), ALT (16.9 U/L), bilirubin (0.5 mg/dL) and folate levels (14.7 ng/mL) were all within the normal ranges and were not statistically different among the three treatment groups.

3.2 | Distribution of Genotypes and Folate Levels According to Treatment Groups

The prevalence of MTHFR polymorphisms at positions 677 and 1298 and DHFR genotype is shown in Table 2 according to treatment groups. Across all 30 participants who had follow-up folate levels, the distribution of each polymorphism in MTHFR 677 was (CC, 43%; CT, 57%; TT, 0%), and the distribution of each polymorphism in MTHFR 1298 was (AA, 53%%; AC, 47%; CC, 0%). Across all treatment groups, deletion of −19 bp of DHFR was 23% (wild type), 10% (homo), and 67% (hetero).

The average serum folate concentrations in participants across treatment groups did trend lower while on treatment. Specifically, the mean serum folate level of participants at screening and visit 4, respectively, was 14.2 and 11.6 ng/L in the EGCG alone group, 16.0 ng/L and 12.0 ng/L in the EGCG and clomiphene group, and 11.6 and 10.3 ng/L in the EGCG and letrozole group. In all three treatment arms, no participants had folate levels below the normal range. The absolute changes in folate levels by genotype are shown in Table 3.

3.3 | Folate Levels According to MTHFR 677, MTHFR 1298 Genotype

Participants were genotyped for MTHFR C677T and stratified by treatment groups, MTHFR 1298 genotype, and DHFR insertion/deletion polymorphism to analyze absolute changes in folate levels in response to treatment. The change in folate levels was similar between the MTHFR C677T wild type (WT) group and MTHFR C677T heteros group ($p = 0.37$). MTHFR 1298

TABLE 1 | Demographic characteristics of randomized patients.

	EGCG	EGCG + clomiphene	EGCG + letrozole	All	*p*-value for comparison among three groups
Participants	12	8	10	30	
Age (years)	30.4 ± 6.7, 30.0 (24.5–36.5)	26.3 ± 6.3, 28.0 (19.0–31.0)	29.5 ± 7.3, 27.0 (25.0–37.0)	28.8 ± 6.7, 28.0 (23.0–35.0)	0.353
BMI (kg/m^2)	12 26.1 ± 6.3, 24.9 (22.2–29.3)	10 27.2 ± 5.9, 25.9 (22.4–31.0)	8 27.7 ± 10.1, 25.0 (21.6–29.8)	30 26.9 ± 7.1, 25.0 (22.3–30.5)	0.882
Hip circumference (cm)	12 114.8 ± 39.9, 104.4 (95.5–112.5)	9 102.2 ± 18.6, 102.0 (90.0–114.5)	8 102.5 ± 20.1, 102.8 (86.8–112.5	29 107.5 ± 29.4, 102.0 (92.0–114.3)	0.553
Waist circumference (cm)	12 92.2 ± 29.0, 81.6 (75.5–99.0)	9 84.8 ± 13.6, 85.5 (75.0–96.5)	8 86.1 ± 23.7, 84.1 (65.5–97.3)	29 88.2 ± 23.1, 83.2 (74.0–96.5)	0.749
Ethnicity					
Hispanic or Latino	1/12 (8.3%)	2/10 (20.0%)	2/8 (25.0%)	5/30 (16.7%)	0.580
Non-Hispanic	11/12 (91.7%)	8/10 (80.0%)	6/8 (75.0%)	25/30 (83.3%)	
Race					
White	8/12 (66.7%)	6/10 (60.0%)	4/8 (50.0%)	18/30 (60.0%)	0.961
Black	1/12 (8.3%)	1/10 (10.0%)	1/8 (12.5%)	3/30 (10.0%)	
Asian	2/12 (16.6%)	2/10 (20.0%)	2/8 (25.0%)	6/30 (20.0%)	
Native Hawaiian/Pacific Islander	1/12 (8.3%)	0/10 (0.0%)	0/8 (0.0%)	1/30 (3.3%)	
I prefer not to answer	0/12 (0.0%)	1/10 (10.0%)	1/8 (12.5%)	2/30 (6.7%)	
Have ever been diagnosed with fibroids	2/12 (16.7%)	1/10 (10.0%)	1/8 (12.5%)	4/30 (13.3%)	1.000
Have had endometriosis	0/11 (0.0%)	0/9 (0.0%)	0/8 (0.0%)	0/28 (0.0%)	NA
History of smoking					
Never	10/12 (83.3%)	8/9 (88.9%)	7/8 (87.5%)	25/29 (86.2%)	1.000
Current	1/12 (8.3%)	0/9 (0.0%)	0/8 (0.0%)	1/29 (3.5%)	
Former	1/12 (8.3%)	1/9 (11.1%)	1/8 (12.5%)	3/29 (10.3%)	

(Continues)

TABLE 1 | (Continued)

	EGCG	EGCG + clomiphene	EGCG + letrozole	All	*p*-value for comparison among three groups
History of alcohol use					
Never	1/12 (8.3%)	1/10 (10.0%)	2/8 (25.0%)	4/30 (13.3%)	0.617
Current	10/12 (83.3%)	9/10 (90.0%)	5/8 (62.5%)	24/30 (80.0%)	
Former	1/12 (8.3%)	0/10 (0.0%)	1/8 (12.5%)	2/30 (6.7%)	

Abbreviations: BMI, body mass index; EGCG, epigallocatechin gallate; NA, not applicable.

TABLE 2 | The frequencies of the MTHFR C677T, MTHFR A1298C, and −19 bp DHFR genotypes.

Gene	Cases *n* (%)
MTHFR C677T	
CC ref	13 (43)
CT	17 (57)
TT	0 (0)
CT + TT	17 (57)
Total	30 (100)
MTHFR A1298C	
AA ref	16 (53)
AC	14 (47)
CC	0 (0)
AC + CC	14 (47)
Total	30 (100)
DHFR −19 bp del	
WT ref	7 (23)
Homo	3 (10)
Hetero	20 (67)
Homo+Hetero	23 (77)
Total	30 (100)

genotypes and DHFR insertion/deletion polymorphisms were also similar among the three treatment groups (Table 3). There was no significant difference between the MTHFR 1298 WT group and MTHFR 1298 Hetero group as well as the stratified subgroups. However, the mean serum folate level significantly decreased ($p = 0.03$) in the group taking EGCG and clomiphene citrate, but was unaltered in the other two treatment groups.

3.4 | Folate Levels According to DHFR Genotype

Participants were genotyped for the highly prevalent insertion/deletion DHFR polymorphism of −19 bp in intron 1. This particular polymorphism has a frequency of the del/del genotype between 10.5% and 48% in various populations [12–16]. In the current study, the DHFR genotype frequency was 23% (WT), 10% (homo; −19 bp deletion), and 67% (hetero; −19 bp deletion) across all treatment groups. The change of folate levels was similar between the DHFR wild-type group and DHFR hetero or homo groups, as well as in the stratified subgroups (Table 3).

4 | Discussion

These results add to the understanding of the safety of EGCG regarding its effects on folate levels. The rationale for the current study was based on previous reports suggesting that EGCG may deplete serum folate levels in human subjects of specific populations, including elderly men, postmenopausal women, and

TABLE 3 | Absolute change in folate levels from screening to visit 4 by genotype.

	MTHFR677-WT	MTHFR677-hetero	*p*
Absolute change in folate levels from screening to visit 4, ng/mL, mean ± SD (*n*)	−3.2 ± 4.1 (*n* = 13)	−2.0 ± 3.0 (*n* = 17)	0.37
EGCG alone group	−3.8 ± 2.6 (*n* = 6)	−1.4 ± 2.1 (*n* = 6)	0.11
EGCG with clomiphene group	−5.0 ± 5.9 (*n* = 4)	−3.1 ± 3.5 (*n* = 4)	0.59
EGCG with letrozole group	0.3 ± 2.7 (*n* = 3)	−2.0 ± 3.5 (*n* = 7)	0.33
MTHFR1298-WT	−1.8 ± 3.5 (*n* = 7)	−1.2 ± 2.8 (*n* = 9)	0.69
MTHFR1298-hetero	−4.9 ± 4.5 (*n* = 6)	−3.0 ± 3.0 (*n* = 8)	0.38
DHFR-WT	−5.5 ± 6.6 (*n* = 3)	−2.8 ± 1.8 (*n* = 4)	0.45
DHFR-hetero or homo	−2.5 ± 3.3 (*n* = 10)	−1.8 ± 3.3 (*n* = 13)	0.61
	MTHFR1298-WT	**MTHFR1298-hetero**	***p***
Absolute change in folate levels from screening to visit 4, ng/mL, mean ± SD (*n*)	−1.5 ± 3.0 (*n* = 16)	−3.8 ± 3.7 (*n* = 14)	0.07
EGCG alone group	−3.7 ± 2.9 (*n* = 4)	−2.1 ± 2.5 (*n* = 8)	0.35
EGCG with clomiphene group	−1.4 ± 2.3 (*n* = 5)	−8.4 ± 4.3 (*n* = 3)	0.03
EGCG with letrozole group	−0.2 ± 3.2 (*n* = 7)	−3.8 ± 2.4 (*n* = 3)	0.12
MTHFR677-WT	−1.8 ± 3.5 (*n* = 7)	−4.9 ± 4.5 (*n* = 6)	0.20
MTHFR677-hetero	−1.2 ± 2.8 (*n* = 9)	−3.0 ± 3.0 (*n* = 8)	0.22
DHFR-WT	−2.1 ± 0.4 (*n* = 2)	−4.7 ± 5.0 (*n* = 5)	0.52
DHFR-hetero or homo	−1.4 ± 3.2 (*n* = 14)	−3.3 ± 3.0 (*n* = 9)	0.16
	DHFR-WT	**DHFR-hetero or homo**	***p***
Absolute change in folate levels from screening to visit 4, ng/mL, mean ± SD (*n*)	−3.9 ± 4.3 (*n* = 7)	−2.1 ± 3.2 (*n* = 23)	0.24
EGCG alone group	−1.0 ± 0.8 (*n* = 2)	−2.9 ± 2.8 (*n* = 10)	0.37
EGCG with clomiphene group	−6.5 ± 5.9 (*n* = 3)	−2.5 ± 3.5 (*n* = 5)	0.26
EGCG with letrozole group	−3.0 ± 0.8 (*n* = 2)	−0.9 ± 3.7 (*n* = 8)	0.47
MTHFR677-WT	−5.5 ± 6.6 (*n* = 3)	−2.5 ± 3.3 (*n* = 10)	0.30
MTHFR677-hetero	−2.8 ± 1.8 (*n* = 4)	−1.8 ± 3.3 (*n* = 13)	0.61
MTHFR1298-WT	−2.1 ± 0.4 (*n* = 2)	−1.4 ± 3.2 (*n* = 14)	0.77
MTHFR1298-hetero	−4.7 ± 5.0 (*n* = 5)	−3.3 ± 3.0 (*n* = 9)	0.54

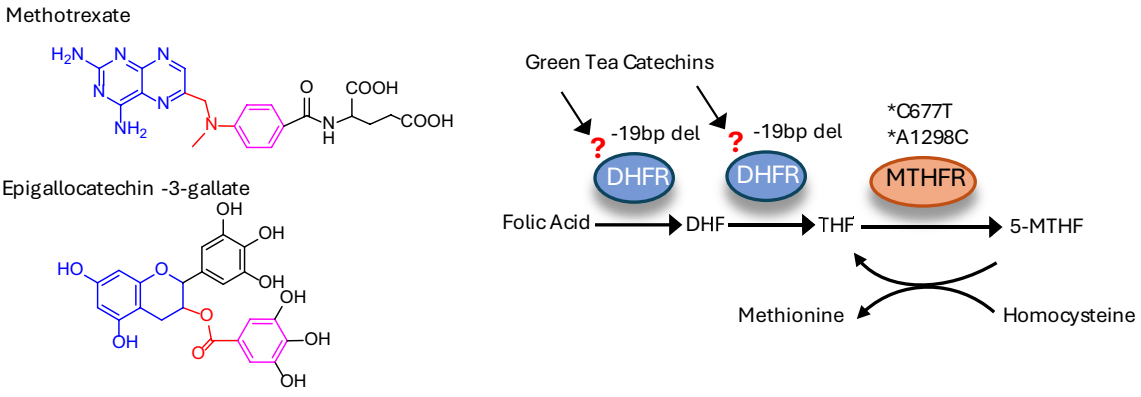

FIGURE 3 | Chemical structure of EGCG and methotrexate and mechanism of how antifolates disrupt dihydrofolate reductase (DHFR).

individuals with other liver-related comorbidities. The current study addressed a weakness of prior epidemiological studies by using a highly characterized green tea extract for treatment and determining participants MTHFR and DHFR genotypes. Polymorphisms of MTHFR are known to decrease enzymatic activity in converting folic acid to folate. For example, a heterozygous mutation of MTHFR 677CT/1298AC genotype has been reported to result in 36% activity compared to wild-type MTHFR [8]. Our data suggest that >75% of participants in our cohort, based on MTHFR and DHFR genotype, may have reduced activity in the folate synthesis pathway, suggesting an increased risk of folate deficiency. In participants with the MTHFR genotype, potential inhibition of DHFR by administration without folic acid supplementation may theoretically increase the risk of developing folic acid deficiency. However, none of the participants had folate levels below the normal range (<4 ng/mL) during the study, and absolute changes in folate remained unaltered in participants with the MTHFR or DHFR genotypes in all three treatment groups. Interestingly, participants who were heterozygous for the MTHFR A1298C genotype exhibited a significant decrease in absolute folate levels over 30 days from the time of the screening visit, despite having normal folate levels before and after treatment.

It is important to note that folate levels continued to remain >4 ng/mL. Folate levels of patient data are shown in Table S1. Furthermore, reports of EGCG-induced DHFR inhibition are based on in vitro studies (Figure 3) [20, 21]. However, EGCG is the ester of epigallocatechin and gallic acid, and ester bonds in blood may lack stability due to the presence of esterases present in vivo. Thus, in vitro assays lacking esterases may not predict what occurs in vivo. Taken together, the current study suggests that EGCG administration for 30 days to individuals at high risk of reduced folate levels did not result in folate deficiency.

Processing of folate utilizes key enzymes including MTHFR and DHFR. DHFR maintains intracellular levels of tetrahydrofolate and provides precursors of purines and pyrimidines involved in the synthesis of DNA, RNA, and amino acids. DHFR is the target of the anti-cancer drug MTX and the anti-bacterial drug TMP [22]. Previous reports suggest that MTX and TMP may share some chemical similarities to green tea catechins [20]. In preclinical studies, EGCG and ECG inhibit DHFR in vitro at concentrations suggesting inhibition of DNA synthesis. Clinically, DHFR inhibitors have the potential to block thymidylate synthesis and de novo purine synthesis, leading to folate deficiencies. Reduction in folate levels in pregnant women is especially concerning due to the risk of neural tube defects during embryonic development. Population-based studies by Matsuzaki in 2008 and Shiraishi in 2010 suggested that high tea consumption in pregnant women may be associated with lower serum folate concentrations [23, 24]. More recently, however, Yazdy et al. [25] reported no association between tea consumption and the neural tube defect spina bifida.

Strengths of our report are that it addresses several major limitations in previous epidemiological studies evaluating reduced folate levels with green tea extract consumption. First, the composition of the tea consumed, including the quantity of green tea catechins, is unknown in these studies. This is important because the source of the plant material, as well as the growing conditions, will have a direct impact on the overall catechin composition [26]. Second, *Camellia sinensis* is used to make different teas such as green tea, oolong tea, white tea, and black tea, each of which has a different phytochemical composition. Third, the dose of tea is based on a diet surveys that stratify dose based on the average number of "cups" of green tea consumed each day. These surveys provided ranges at best but did not provide a specific dose of the green tea catechins. Fourth, these studies did not consider genetic polymorphisms of key enzymes in the folate pathway present in the populations studied. This is important because specific polymorphisms of MTHFR are associated with lower folate levels, precluding stratification of subjects based on polymorphism genotyping during data analysis.

The current study has a few limitations. Participants received EGCG for ~30 days while typical fertility-promoting regimens involving clomiphene or letrozole may require several ovulatory cycles (i.e., 60 to 120 days) to achieve conception. In this Phase 1 safety trial, all participants were required to use barrier contraception while taking EGCG, and no participants used folate supplementation. In the FRIEND trial, which targets women with uterine fibroids and infertility, participants will take EGCG and folic acid supplementation until pregnancy is achieved. The FRIEND study will be evaluating liver function and folate levels over 4 months to address the limitations of the current study while accounting for the folic acid supplementation. From a practical point of view, folic acid supplementation during EGCG administration while undergoing a fertility-promoting regimen will further decrease the likelihood of developing folate deficiency. In fact, folic acid supplementation with weekly green tea drinking during the preconception period was not associated with an increased risk of neural tube defects [27].

Author Contributions

J.J.J., H.S., A.A.-H., J.H.S., F.G., B.S., S.A.C., G.M.C., H.H., and H.Z. wrote the manuscript; J.J.J., H.S., A.A.-H., J.H.S., F.G., H.S.T., and B.S. designed the research; J.J.J., H.S., A.A.-H., J.H.S., F.G., H.S.T., B.S., H.H., and H.Z. performed the research; J.J.J., H.S., B.D., H.H., and H.Z. analyzed the data; and J.J.J., H.H., and H.Z. contributed new reagents/analytical tools.

Ethics Statement

The study was conducted in accordance with the Declaration of Helsinki and approved by the Institutional Review Board of Johns Hopkins University (IRB00235806, first approved in November 2019).

Consent

Informed consent was obtained from all subjects involved in the study. Written informed consent has been obtained from participants to publish this paper.

Conflicts of Interest

A.A.-H. is serving as a consultant for OBS-EVA, Myovant, Pfizer, Bayer, and previously for AbbVie, Novartis, and Crila. J.J.J. is serving as a consultant for Wholesome Nutritionals. The other authors declare no conflicts of interest.

Data Availability Statement

Raw data were generated at the Data Consortium Center at Yale University. Derived data supporting the findings of this study are available from the corresponding author on request. The data are not

publicly available due to containing information that could compromise the privacy of participants.

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Supporting Information

Additional supporting information can be found online in the Supporting Information section.