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## Response of serum and red blood cell folate concentrations to folic acid supplementation depends on methylenetetrahydrofolate reductase C677T genotype: Results from a crossover trial

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### Abstract

**Scope**—By increasing blood folate concentrations, folic acid supplementation reduces risk for neural tube defect-affected pregnancies, and lowers homocysteine concentrations. We assessed response of red blood cell (RBC) and serum folate to folic acid supplementation, and examined association of response with the genetic polymorphism C677T of the methylenetetrahydrofolate NAD(P)H (*MTHFR*) gene.

**Methods and Results**—Randomized, controlled, crossover trial with two folic acid supplement treatment periods and a 30-week washout period. The primary outcome is blood folate (serum and RBC) concentrations. Volunteers (n=142) aged 18-69 were randomized to two of three doses (0, 200, and 400 µg) of folic acid for twelve weeks. Serum folate response depended on treatment period with significant responses to 200 µg seen only in the second treatment periods (4.4 ng/mL or 3.4 ng/mL). Additionally, serum folate increased as folic acid dose increased to 400 µg (p< 0.01) and response was greater after the washout period (8.7 ng/mL), than after a 6-week run-in

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**CONFLICT OF INTEREST STATEMENT** Drs. Anderson, Beresford, Lampe, Deeb, Feng and Motulsky, and Mr. McLerran declare they have no financial or personal relationships between themselves and others that might bias their work. There are no conflicts of interest.

(2.3 ng/mL). The differential change attributable to a daily supplement of 400 µg compared to 200 µg was 96.8 ng/mL; while the change attributable to 400 µg compared to 0 µg was 121.4. Increases in RBC folate concentrations with 400 µg occurred within *MTHFR* gene mutation (C677T); and in the African American group.

**Conclusions**—Serum folate concentration is responsive to modest increases in folic acid intake. Red blood cell folate increases only with higher additional doses of folic acid supplementation, and this is true for each *MTHFR* C677T genotype.

### Keywords

Folic acid; fortification; methylenetetrahydrofolate reductase; supplementation

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## INTRODUCTION

Low blood folate concentration is associated with low dietary intake of folic acid [1], and with the homozygous state of thermolabile variant (C677T) of the methylenetetrahydrofolate reductase NAD(P)H (*MTHFR*) gene [2-4]. By increasing blood folate concentrations, folic acid supplementation reduces homocysteine concentrations [5, 6], markedly reduces the frequency of neural tube defect-affected pregnancies [7, 8], hearing loss among the elderly [9], and possibly cognitive decline [10-13]. Contrary to earlier hypotheses, folic acid supplementation does not reduce risk of cancer [14-17], or cardiovascular diseases (CVD) [17-20].

The *MTHFR* gene has received attention as a factor that might modulate the risk relationship between folic acid intake and CVD [21-23]. The enzyme methylenetetrahydrofolate reductase catalyzes the reduction of 5, 10 –methylene tetrahydrofolate to 5-methyltetrahydrofolate, thus transferring a methyl group to cobalamin, which in turn donates a methyl group for the conversion of homocysteine to methionine. The *MTHFR* gene has many single nucleotide polymorphisms (SNPs) and the C677T (rs 1801133) is one of these SNPs. The thermolabile (C677T) allele is characterized by a point mutation at position 677 of the *MTHFR* gene that converts cytosine (C) into a thymine (T) resulting in an amino acid substitution (alanine to valine) in the enzyme. The 677C allele of *MTHFR* has been shown to have higher enzyme activity than the 677T allele in vitro [24]. *MTHFR* C677T has three genotypes: 677CC is normal or wild-type, 677CT is the heterozygote, and 677TT is the mutant. The *MTHFR* 677TT genotype occurs with a frequency of 5 to 10% in U.S. Caucasian populations and the basic molecular defect can be identified in humans. Recently it has been documented that individuals with 677TT genotype have elevation in plasma homocysteine concentrations that is not correctable with folic acid supplementation [23], in contrast to earlier reports [25, 26].

Prior to folic acid fortification of foods which was initiated in 1998 in the United States (US), folate deficiency was common in some Western populations, especially in subgroups of populations such as older adults, with estimates documented as high 40% [27]. In the Netherlands, Belgium and Germany estimates of folate deficiency have been 30-50% [28].

Although folic acid supplementation is generally considered a simple, cost-effective approach to improving blood folate status, the nature of the relationship between folate intake and serum and red blood cell folate needs to be understood. The response of folate status to folic acid supplementation should be characterized; and the amount of folic acid intake necessary to maintain adequate folate status in healthy individuals should be estimated, as it cannot be assumed that the relationship between folate intake and serum or RBC folate is quantitative and linear.

The aims of this investigation are to compare changes in red blood cell folate and serum folate status in response to modest change in folic acid intake, to estimate the amount of time required following folic acid supplementation for serum and RBC folate concentrations to return to those observed at a baseline visit, and to examine the association of response with the *MTHFR C677T* genotype.

## MATERIALS AND METHODS

### Study Design

The study was designed to be a randomized, double-blind, controlled, crossover study of different doses of folic acid supplementation. Randomization to study and first dose assignment was within one of four strata, defined by *MTHFR C677T* genotype and African American race. A separate stratum was created for African Americans because of known differences in frequency of *MTHFR C677T* genotypes than other race-ethnic groups. In order to keep the strata of similar size, we screened a large number of volunteers and used a different sampling fraction for each stratum for conditional invitation to join the randomized study. Therefore, there was an initial screening visit, which was followed by a 6-week run-in period, followed by a 54-week main study period.

As soon as there were at least six members of a group, individuals were scheduled for a visit to the UW's Clinical Research Center. At this visit, eligibility was checked, the study was explained again, and placebo pills were dispensed for the 6-week run-in period. The purpose of this initial run-in period was to assess compliance with study procedures and to provide a washout for previous vitamin use. Adequate compliance was defined as missing no more than one study pill per week during run-in. At the end of the run-in period, those with adequate compliance were invited to continue with the 54-week long study. These 54 weeks included a baseline visit, a 12-week intervention period (first period), a 30-week washout period (i.e., cessation of intervention folic acid supplementation) and another 12-week intervention period (after washout). Two doses - 200 µg and 400 µg - of folic acid were evaluated in the two parallel randomized studies.

Those invited to continue were asked to maintain their usual diets and lifestyle, to report any illnesses over the study period, and to abstain from additional folic acid supplements and foods fortified with 100% of the recommended dietary allowance of folic acid during the entire study. Foods fortified with 100% of the recommended dietary allowance included breakfast cereals and cereal bars.

The folic acid doses used in the study were selected to correspond with folic acid supplementation strategies that were hypothesized to reduce total homocysteine concentrations. The 200 µg dose is similar to the folic acid intake accrued from two servings of folic acid fortified cereal or grain product in the US, while the 400 µg dose is similar to the amount of folic acid obtained in a regular dietary supplement. The trial was conducted in Seattle, Washington at the University of Washington (UW) from 1998-2001 - i.e. after folic acid fortification began in the U.S. The study protocol was approved by the UW Human Subjects Review Board, and volunteers signed informed consent forms.

### Study Participants

All interested volunteers were interviewed by phone to determine eligibility for the study. Participants were eligible if they were between the ages of 18 and 69 years, willing to take only the folic acid supplements provided by the study for 54 weeks, not pregnant or planning a pregnancy, free from chronic disease or diseases known to interfere with folate metabolism, and not taking medications that may decrease folic acid absorption or utilization such as methotrexate, bactrim, and phenobarbital. All volunteers who met study eligibility criteria were screened for the thermolabile variant C677T genotype of *MTHFR* and for plasma homocysteine concentrations. Homocysteine concentrations were analyzed by the Clinical Nutrition Research Unit laboratory at the University of Washington, using a modification of the method employing monobromobimane derivatization and high-performance liquid chromatography. Briefly, 30 µl of plasma was mixed with internal standard (cysteamine) and then reduced with NaBH<sub>4</sub>. The resulting mixture was derivatized with monobromobimane in nethylmorpholine buffer; the reaction was terminated with glacial acetic acid. The derivatized thiol compounds are stable for at least one week after derivatization. After standing on ice, protein precipitate was removed, and 20 µl of the sample was injected on to a 150 × 4.6 mm Hypersil ODS column equilibrated with an ammonium formate buffer, pH 3.2. The chromatogram is developed by an acetonitrile gradient, and derivatized thiols are detected by fluorescence. Collection and analysis of data were computerized, using the EZChrom chromatography data system. The laboratory runs high and low control samples for each batch of assays as part of their in-house quality control procedures. For homocysteine, the between run coefficients of variation were 8.6% and 7.3% for high and low control samples respectively; the intra-assay CVs were 6.4% and 3.7% respectively (n=20).

Participants were assigned to one of four groups: 677CC, 677CT, 677TT genotype (if race was other than African American), or African American. Sampling fractions were applied to the available volunteers to enroll 60 individuals classified as 677CC, 60 as 677CT, 40 as 677TT and 40 African Americans for a recruitment goal of 200 participants.

### Randomization and Supplement Doses

Stratified random allocation was used to assign participants to dose trial and within trial to treatment order. Strata were defined by *MTHFR* C677T genotype, African American and low or high homocysteine concentrations (<10 µmol/L or ≥10 µmol/L). Three identical pills were formulated by Tishcon Corporation containing 0, 200 and 400 µg folic acid respectively. One of the dose trials compared 0 µg (placebo) with 200 µg folic acid. The

other dose trial compared 200 µg with 400 µg folic acid. Each participant was randomly assigned to one of the two trials; and to one dose order such that they received two treatments with a washout period in between.

At the baseline clinic visit, a fasting blood sample was drawn to provide baseline concentrations of serum and RBC folate. A 45-day supply of folic acid pills was given to volunteers, and a follow-up appointment was scheduled for 6 weeks later. Three weeks after the baseline study visit, the volunteer was called by the study coordinator to make sure that study pills were being taken, and to answer study-related questions. Compliance with study pills was determined by counting the contents of the bottles brought back to the clinic at the end of the 6-week period.

Additional clinic visits for fasting blood draws occurred at weeks 6, 12, 18, 24, 30, 36, 42, 48 and 54. Each treatment period lasted 12 weeks, and was characterized by measures at the start, the middle and the end of the period. The study protocol for each participant visit was therefore 12 weeks of treatment one, 30 weeks of washout, and 12 weeks of treatment two.

### Statistical Methods

Statistical analyses were conducted using Statistical Analysis System software (version 9.1; SAS Institute, Inc.). Descriptive statistics (means and percent) were used to describe baseline participant characteristics. Paired t-test analyses were used to compare the mean effect of the three doses within and between dose trials. Analysis of variance compared mean changes in folate status during treatment within each dose trial by treatment group and treatment order. Where significant differences were found by treatment order, estimates from the two periods could not be combined. Where no significant differences were found, estimates of the same dose effect were combined to increase power. In analyses within *MTHFR* C677T genotype, African Americans were excluded, and results for African Americans are reported separately since the 677TT genotype occurred in less than 10% of this group. P values <0.05 were considered significant.

## RESULTS

These analyses include 142 male and female volunteers. Of the 707 participants screened, 238 were randomized (Figure 1). Volunteers ranged in age from 18-69 years. Overall, the mean age of participants was 42 years, 46.7% were male, 74.7% were white, and the mean body mass index (BMI) was 27.4 kg/m<sup>2</sup>. As shown in Table 1, there were no significant differences among the four groups with respect to age, gender or baseline concentrations of serum and RBC folate. Group B had a slightly higher percentage of 677TT genotype and Group C had a slightly lower percentage of African American participants.

Participant compliance was determined to be good on the basis of pill counts. Most (85.3%) volunteers were high compliers and on average forgot to take no more than one pill per week during the entire study period. Poor compliance was defined as missing three or more pills per week. There were no participants with poor compliance based on pill counts. The CV for serum folate was about 0.25 and for RBC folate, about 0.35.

Serum folate varied by period endpoints and treatment group in the first treatment period, but variations between the groups had disappeared by the end of washout. As shown in Table 2, serum folate response to folic acid depended on treatment period with significant responses to 200 µg seen only in the second treatment periods (4.4 ng/mL or 3.4 ng/mL). Supplementation with 400 µg resulted in an increase ( $p < 0.01$ ) in serum folate in each treatment period but the magnitude of response was greater after the 30-week washout (8.7 mg/mL) than in the first treatment period (2.3 ng/mL).

There was an increase in RBC folate concentrations after supplementation with active doses of folic acid for twelve weeks (Table 3). The RBC folate response did not depend on treatment period and response was seen in both periods. Table 4 shows the effect of different doses of folic acid, with groups combined, on RBC folate. When the effect estimates are combined, the differential change attributable to a daily supplement of 400 µg compared to 200 µg is 96.8 ng/mL ( $p < 0.01$ ) and the differential change attributable to a dose of 400 µg compared to placebo is 121.4 ng/mL. As shown in Table 5, the increases in RBC folate concentrations seen with 400 µg folic acid are found within each *MTHFR* C677T genotype, with individuals with 677TT genotype having mean (95%CI) response [246.6 ng/mL (139.0, 354.1)], apparently larger than those with 677CC [(72.2 (3.4, 141.0)] or 677CT [(87.6 (24.3, 150.9)]. For African Americans, a significant response in RBC folate concentration was seen with supplementation with 400 µg only (Table 5). The results for African Americans are not divided by genotype, and 15 of them were 677CC, 7 were 677CT and 0 were 677TT in our dataset.

## DISCUSSION

In this folic acid intervention study, using a crossover design, we observed that blood folate status can be improved with modest doses of folic acid supplements. Significant responses in serum folate concentrations were seen after supplementation with 200 µg and 400 µg. RBC folate concentrations increased after taking 400 µg, but were not responsive to 200 µg. Increases in RBC folate concentrations seen with 400 µg depended on *MTHFR* C677T genotype with 677TTs having a larger response than 677CCs or 677CTs.

Our findings of an increase in serum and RBC folate with increasing folic acid intake are consistent with results from observational studies as well as randomized clinical trials. Serum folate has been shown to be highly influenced by recent intake, peaking two to three hours after a load in most people [29, 30]. Red cell folate, however, reflects an integrated measure of intake over about 120 days, because this is the life of a red blood cell and the folate content is set before the erythrocyte (red cell) leaves the bone marrow [29, 31]. Furthermore, data from the Framingham Offspring Study cohort showed increased mean serum folate concentrations in middle-aged and older adults consuming cereal-grain products fortified with folic acid [1]. Likewise, comparisons between 1988–1994 National Health and Nutrition Examination Survey (NHANES) III data and 1999 NHANES – collected after folic acid fortification of the food supply—showed increased serum and RBC folate concentrations among women of childbearing age [32]. These early NHANES findings were corroborated by later NHANES reports [33]. Additionally, trials conducted in



several parts of the world consistently documented increases in serum folate concentrations after intervention with folic acid fortified foods [34-37] or supplements [34, 38-42].

Our finding that serum folate response depends on treatment period might be because folate concentrations at week 42 of our study reflect a more accurate non-supplemented measure than folate concentrations at week 0. It is possible that the six week run-in period may not have been long enough to washout any prior folic acid supplementation, such that serum folate response in the first treatment period may have been mis-estimated. The use of folic acid containing vitamins among study participants varied at week 0, but was apparently washed out by week 42 (30 weeks after the cessation of supplements). It has been documented that the magnitude of change in blood folate concentrations relative to baseline will be influenced by the initial status of the subjects [37]. In our study, the comparability of baseline folate status of individuals in each folate treatment group reduces the potential influence of difference in folate body stores on folate response. Our finding that RBC folate concentrations increased after taking 400 µg, but were not responsive to 200 µg suggests that intake of up to 200µg may not appreciably change the longer term measure of folate status reflected in RBC folate.

Our findings regarding the association of folic acid response with *MTHFR* C677T genotype are generally consistent with other published reports [23, 43-45]. A large cross-sectional analysis of data from a Dutch cohort revealed that at any folic acid intake level, individuals with 677TT genotype had lower plasma folate concentrations than those with 677CT or 677CC [44]. Further, our findings are similar to results from a folic acid exclusion study that tested the effect of a folic acid exclusion diet versus a folate-rich diet versus a folic acid supplement showed that plasma folate was higher after folate supplementation [43], and there was no difference in response between genotypes [43]. In another trial that evaluated the effect of daily supplementation for two months of 0.5 mg folic acid on serum and red cell folate concentrations, individuals with 677TT genotype initially had the lowest absolute increase in serum folate concentration [45], however, after four months of supplementation, additional increases were seen in serum and red cell folate and there were no significant differences among the three genotype groups. We found that increases in RBC folate concentrations after 400 µg/day of supplements depend on *MTHFR* C677T genotype with 677TTs having a larger estimated response than 677CCs or 677CTs. However, because the differences were not statistically significant, we use caution in interpreting this finding.

There are limitations and strengths of the study worth noting. We may not have been able to fully characterize the response to folic acid supplementation for the *MTHFR* C677T genotypes because of a limitation of the folate assay. The assay we used for quantification of blood folate concentrations is unable to distinguish between different folate vitamers such as methyl tetrahydrofolate, formyl tetrahydrofolate, and others. Folate vitamers distribution in the blood may differ among the *MTHFR* C677T genotypes and the vitamers are not equally detected by the assay. Also, our results have limited generalizability, and should be applied to populations with folic acid fortification and the resultant higher concentrations of blood folate. Strengths of this study include its randomized, controlled, crossover design; and relatively long intervention and washout periods.

This study protocol involved the assessment of blood folate status over a 54-week period in response to folic acid supplementation with 0, 200, or 400 µg/day. An important aspect of the supplement doses used is that they are comparable to two common public health strategies to improve folic acid consumption. The 400 µg/day dose represents the use of vitamin supplements containing folic acid, and the 200 µg/day dose represents the consumption of foods fortified with folic acid. Our results suggest that daily supplementation with 400 µg resulted in blood folate concentrations that were almost twice as high as folate concentrations after daily supplementation with 200 µg. The results of this study suggest that vitamin supplements that contain 400 µg of folic acid provide a greater blood folate response than the intake of 200 µg/day folic acid from folic acid-fortified foods. However, additional food fortification with low doses of folic acid, or behavioral change to increase the servings of folate-fortified foods may be good options to achieve moderate increases in blood folate status.

The results of this study suggest that moderate doses of folic acid can provide change in folate status. This is important given the observed relationships of low folate status with neural tube defect affected pregnancies and elevated homocysteine concentrations. Persons at risk for these conditions can expect an increase in serum folate concentrations after supplementation with 200 µg folic acid, and an increase in red blood cell folate after supplementation with 400 µg.

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## List of abbreviations

**MTHFR**      methylenetetrahydrofolate reductase gene

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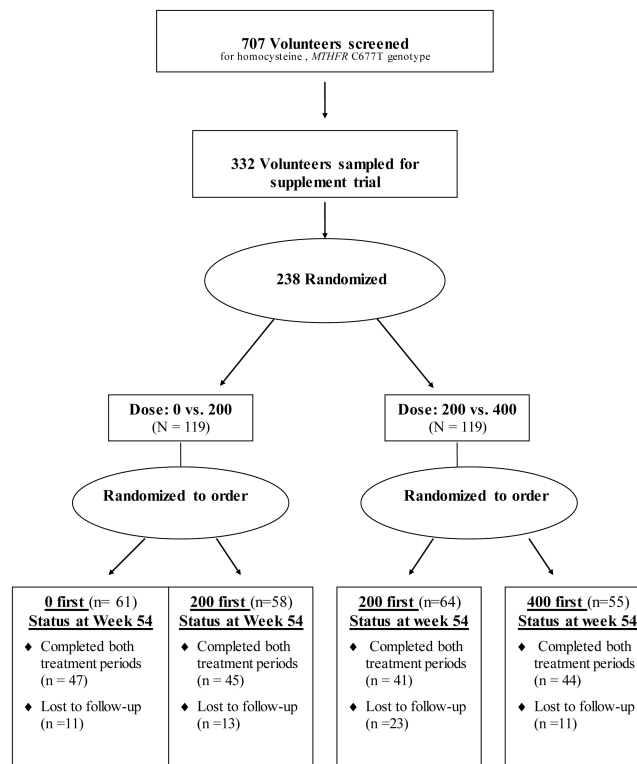
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**Figure 1.**  
Flow diagram of progress through study phases

**Table 1**

Baseline characteristics of study population

	Treatment Group*			
	A n=42	B n=34	C n=34	D n=32
Mean Age (y)	40.9	39.1	43.1	44.4
Gender (% male)	45.2	47.1	44.1	40.6
Mean Baseline Serum Folate (ng/mL)	13.3	13.8	14.3	13.9
Mean Baseline RBC Folate (ng/mL)	515	562	520	517
% 677TT Genotype	14.3	23.5	17.6	9.4
% African-American	16.7	20.5	5.9	18.8

\* A=0 and 200 µg/day, B=200 and 0 µg/day, C=200 and 400 µg/day, D=400 and 200 µg/day

**Table 2**

Serum folate (ng/mL) response to supplementation (mean (s.e.))

	Start	Middle	End	Response estimate at 12 weeks (95%CI)
<b>DOSE 0 µg</b>		<b>0</b>	<b>0</b>	
Treatment Group A First period (n=42)	13.3 (0.37)	13.0 (0.45)	13.0 (0.35)	-0.1 (-1.7, 1.5)
Treatment Group B (After washout) (n=34)	11.6 (0.53)	11.5 (0.73)	11.8 (0.64)	0.4 (-1.4, 2.2)
<b>Dose 200µg</b>		<b>200</b>	<b>200</b>	
Treatment Group B (First period) (n=34)	13.8 (0.46)	14.9 (0.46)	14.9 (0.38)	1.0 (-0.8, 2.8)
Treatment Group C (First period) (n=34)	14.3 (0.38)	15.0 (0.33)	14.5 (0.34)	0.2 (-2.0, 2.4)
Treatment group A (After washout) (n=42)	11.8 (0.42)	15.5 (0.76)	16.2 (1.34)	4.4 (2.8, 6.0) <sup>b</sup>
Treatment Group D (After washout) (n=32)	11.0 (0.49)	13.7 (0.71)	14.2 (0.72)	3.4 (1.2, 5.6) <sup>b</sup>
<b>Dose 400µg</b>		<b>400</b>	<b>400</b>	
Treatment Group D (First period) (n=32)	13.9 (0.49)	15.8 (0.87)	16.1 (0.72)	2.3 (0.1, 4.5) <sup>a</sup>
Treatment Group C (After washout) (n=34)	11.3 (0.47)	17.6 (2.04)	20.0 (1.69)	8.7 (6.7, 10.7) <sup>b</sup>

<sup>a</sup>Significant at p<0.05,<sup>b</sup>Significant at p<0.01



**Table 3**

Red blood cell folate (ng/mL )response to supplementation (s.e.)

	<b>week 0</b>	<b>week 12</b>
<b>DOSE 0 µg</b>		<b>0</b>
Treatment Group A First period (n=42)	516 (29)	505 (27)
Treatment Group B (After washout) (n=34)	586 (33)	571 (34)
<b>Dose 200µg</b>		<b>200</b>
Treatment Group B (First period) (n=34)	562 (34)	584 (29)
Treatment Group C (First period) (n=34)	520 (40)	546 (42)
Treatment group A (After washout) (n=42)	533 (26)	566 (27)
Treatment Group D (After washout) (n=32)	583 (41)	552 (31)
<b>Dose 400µg</b>		<b>400</b>
Treatment Group D (First period) (n=32)	517 (23)	603 (33)
Treatment Group C (After washout) (n=34)	528 (26)	654 (44)

**Table 4**

Effect on RBC folate of different doses of folic acid (combining groups)

Effect	Difference Estimate*	CI	p-value
200 vs. 0	24.6	-4.6, 78.3	0.18
400 vs. 200	96.8 <sup>a</sup>	67.5, 157.4	<.001
400 vs. 0	121.4 <sup>a</sup>	88.2, 210.5	<.001

\* Difference of the mean difference of both doses.

<sup>a</sup>Significant at p<.01

**Table 5**Dose effect estimates (mean (95% CI) on RBC folate by *MTHFR* C677T genotype and race

	0*	200*	400*
<b>677CC</b> (n = 58)	7.3 (-33.9, 48.4)	19.9 (-17.5, 57.3)	72.2 (3.4, 141.0) <sup>a</sup>
<b>677CT</b> (n = 61)	-46.0 (-83.0, -9.1) <sup>a</sup>	10.9 (-23.7, 45.6)	87.6 (24.3, 150.9) <sup>b</sup>
<b>677TT</b> (n = 23)	11.6 (-38.8, 62.0)	-8.5 (-60.8, 43.8)	246.6 (139.0, 354.1) <sup>b</sup>
<b>African Americans</b>	-3.6 (-45.4, 38.2)	11.5 (-5.8, 58.9)	105.6 (55.4, 155.8) <sup>b</sup>

<sup>a</sup>Significant at p<.05<sup>b</sup>Significant at p<.01

\*Randomization groups and time points are combined for each dose level.