Effect of Folic Acid and Vitamin B12 Supplementation on Homocysteine Level in association with MTHFR C677T Polymorphisms in Overweight Female Adults

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Objective: The aim of this study was to evaluate the effect of folic acid and vitamin B12 supplementation on Hcy level based on the MTHFR C677T polymorphisms in Indonesian overweight females.

Methods: The study involved 110 female adults (20±1.3 years) with a body mass index (BMI) above 22.9 (mean: 25.3±2.3). DNA was extracted from saliva in order to identify the SNP of MTHFRC677T using PCR-RFLP. 15 of 110 subjects were chosen to represent all MTHFR genotypes (5 CC, 6 CT and 4 TT) and assigned two different treatments: control (placebo), and supplement (500µg folic acid and 1 mg vitamin B12) for 4 weeks. Hcy levels were measured using direct immunochemical assay.

Result: This study population (n=110) showed that 677CC allele had the highest frequency (67%), followed by 677CT (29%) and 677TT (4%). The results demonstrated no significant differences of Hcy level among all subjects before treatment, but Hcy level was slightly higher in T allele subjects. The folate and vitamin B12 treatment significantly reduced the level of Hcy in supplement group (p = 0.038), but not in placebo group. The difference between placebo and treatment was observed to be the highest in TT, followed by CT and CC subjects.

Conclusion: This study showed that subjects who had T allele, tended to have a higher level of Hcy. Supplementation of folate and vitamin B12 was proven to lower the level of Hcy, and TT subjects were proven to be the most responsive.

1. Introduction

Cardiovascular disease (CVD) is known as the leading cause of death and disability worldwide, including Indonesia. In 2008, an estimated of 17.3 million people died from CVDs and by 2030, it is predicted to be risen over 23 million people. Atherosclerotic heart disease, or widely known as coronary heart disease (CHD) is one of CVD group occupied the first place of cause of death in Indonesia and the number keeps rising over the years [1-3].

There are many factors associated with increased risk of CVD. Some of risk factors are classified as non-modifiable, such as age, sex and genetics; whereas behavioral risk factors, such as physical inactivity, tobacco use, unhealthy diet, and harmful use of alcohol, which are responsible for about 80% of atherosclerotic heart disease, are able to be modified by lifestyle or pharmaceutical intervention. Both of modifiable and non-modifiable factors can lead to intermediate risk factors, such as hypertension, hyperlipidemia, glucose intolerance and overweight or obesity, which can lead to chronic disease development [4].

80-90% of people dying from CHD have one or more major risk factors that are influenced by lifestyle. Among the numerous factors above, overweight is one to be highlighted especially because the incidence is increasing each year. World Health Organization stated that the prevalence of overweight in Indonesian women is
higher than men and also expected to increase higher in women over 10 years [5].

The actual risk factors of CVD (blood pressure, blood lipid profiles, BMI, blood glucose) can be measured, but they do not accurately predict the cardiovascular events. Therefore, several studies over years focused on the identification of novel risk factors. To date, homocysteine (Hcy), a non-protein-forming sulfur amino acid that is synthesized as by-product of methionine metabolism, was promoted to be a novel risk factor for the development of CVD [6, 7].

An elevated Hcy level in the plasma or serum, a condition called hyperhomocysteinemia (HHcy), has been reported to be a significant and independent risk factor for CVD as it can cause multi-disease manifestations for cardiovascular pathogenesis [8]. The concentration of Hcy is determined by both genetic and non-genetic factors. An increase in Hcy level could be influenced by genetic variation that involved in the methionine pathway. The most widely accepted polymorphism is the 677C>T mutation in the MTHFR gene (rs1801133). Evidence suggested that plasma Hcy concentrations were significantly higher in the subject with the 677TT genotype than those with CC or CT genotype [9] and the homozygotism for the MTHFR 677TT polymorphism was associated with an increased risk of atherosclerotic heart disease [10]. Besides, Hcy level could also be influenced by nutrition that is involved in the methionine pathway, including folic acid and vitamin B12. Both supplements were commonly used in trials and studies which were aimed to lower Hcy levels [7, 11].

To date, most studies have assessed the effects of B-vitamins and the correlation of MTHFR C677T polymorphisms on Hcy levels mainly on middle-aged or elderly individuals, with the relation on certain specific diseases. Some studies also reported that differences in gender, age, nutrition status, and BMI were also involved in the prediction of Hcy concentration [12-14]. Accordingly, we decided to focus on certain subjects with specific gender, age, BMI in order to see the relation the genetic and nutrition factor effect only. In this research, we chose Indonesian overweight female adults that considered not developing CVD disease. Therefore, the purpose of this study was to evaluate the effect of folic acid and vitamin B12 before and after supplementation in Indonesian overweight female adult on Hcy level based on carrier status for the MTHFR C677T polymorphisms.

2. Materials and Methods

2.1 Sample Collection

This study included 110 female participants. All participants were Indonesian people who lived in Jakarta and surroundings, aged 18-30 years old, classified with BMI (Body Mass Index) ≥ 22.9 (kg/m²), and had been identified as non-smokers, non-alcohol drinker, non-pregnancy. Saliva was obtained from all participants as the source of DNA. For intervention research, blood samples were collected from fasted subjects (n=16) for determination of biochemical parameter. Hcy concentrations were measured using ADVIA Centaur® System (Siemens, US).

2.2 DNA Isolation

Genomic DNA from saliva was isolated using phenol-chloroform method. Saliva was transferred into micro centrifuge tube, added with Tris EDTA 1x and sodium acetate 0.2M, SDS 10% and Proteinase-K (20mg/ml) were added and the sample was incubated at 56°C for 30 minutes. Samples were added with phenol and chloroform, mixed vigorously and centrifuged at 13800 g for 2 minutes. Supernatant was transferred into new micro centrifuge tube and was added with ethanol absolute, mixed vigorously. The sample was incubated at -40°C for 5 minutes and centrifuged at 13800 x g for 2 minutes. Supernatant was transferred into new micro centrifuge tube and was added with ethanol absolute, mixed vigorously. The sample was incubated at -40°C for 5 minutes and centrifuged at 13800 x g for 2 minutes. Supernatant was discarded and the pellet was added with ethanol 70%, and centrifuged at 13800 x g for 2 minutes. The pellet was centrifuged again without the supernatant, at 13800 x g for 1 minute. Pellet was added with ddH2O and incubated 56°C for 15 minutes. The DNA concentration was measured using NanoDrop 2000 (ThermoScientific).

2.3 MTHFR C677T Mutation Analysis.

The MTHFR C677T (rs1801133) gene mutation was identified by PCR-RFLP methods. Briefly, about 50 to 80 ng DNA samples were amplified in a final volume of 25 µL containing 1x Go Taq Green mastermix buffer, 0.4 µM of each primer. The following sense and antisense primer to amplify SNP rs1801133 were 5'-GCCAGCCACTCAGTTTTA-3' and 5'-AGGACGGTGCGGTGAGAGTG-3' [15]. The PCR reactions were carried out as follows initial denaturation at 95°C for 5 minutes, denaturation at 95°C for 30 seconds, annealing at 62°C for 30
seconds, extension at 72°C for 45 seconds, final extension at 72°C for 5 minutes, total number of cycles: 35. The DNA fragments were visualized on a 2.5% agarose gel followed by staining with ethidium bromide. Analysis was performed at least twice to confirm the genotype.

2.4 Intervention Study

16 of 110 subjects were chosen to represent all the possible MTHFR genotypes (6 subjects with 677CC, 6 subjects with 677CT, 4 subjects with 677TT) and assigned two different treatments: control (placebos) and supplements (500µg folic acid and 1 mg vitamin B12) orally and consecutively for 4 weeks.

2.5 Homocysteine Determination

All blood samples were collected after 12 hours of fasting by venipuncture into a non-additive containing tube. Blood clots and the serum is separated by centrifugation within 1 hour after blood collection. Total serum Hcy was measured with the principle of competitive immunoassay, direct, chemiluminescent technology using ADVIA Centaur® HCY assay (Siemens, US). Both of blood collection and Hcy determination were performed before and after intervention studies in cooperation with Prodia Clinical Laboratorium.

2.6 Statistical Analysis

Statistical analysis was performed using SPSS (version 21.0, IBM, USA). Genotype frequency of MTHFR C677T was analyzed statistically with Hardy-Weinberg predictions. Chi-square test ($\chi^2$) was used to examine the distribution of the different genotypes. Shapiro Wilk tests for normality were performed for all data. The 2-tailed nonparametric Kruskall Wallis and Mann Whitney U-test was used for comparison between groups in order to evaluate the effect of folic acid and vitamin B12 supplementation on Hcy level in each genotype. Significance was assigned at P-value of <0.05.

2.7 Ethics statement

This study was reviewed and approved by Institute of Research and Community Service Atma Jaya Catholic University. Informed written consent was obtained from all participants following the contents of the study.

3. Result

Visualization of PCR-amplified fragment in the gene for MTHFR and the different genotype patterns for C677T MTHFR gene mutation were shown in Fig. 1. The amplified product of 418 bp was digested with HinfI, resulting in 77 and 341 bp bands for the wild genotype (677CC); 77, 165, 176, and 341 bp bands for heterozygous (677CT); and 77, 165, and 176 bp bands for homozygous (677TT). Due to the separation limitation of the agarose gel, 165 and 176 bp band were seen as a single band.

The pre-intervention study involved 110 overweight female adults (mean age: 20±1.3 years; mean BMI: 25.3±2.3) with the distribution of MTHFR genotypes is in agreement with the expected Hardy-Weinberg ratio (p > 0.05). The genotype frequencies were 0.67, 0.29, and 0.04 for C/C, C/T, and T/T carriers, respectively (Fig. 2).

![Figure 1](image1.png)

**Figure 1.** (a) PCR visualization of MTHFR C677T fragment gene. Lane M, marker 100bp DNA Ladder (New England Biolabs); lane N, no template control; lane 1-3, MTHFR C677T fragment gene (400bp). (b) Digestion visualization for MTHFR C677T fragment gene. Lane 1-3: MTHFR 677CC, MTHFR 677CT, MTHFR 677TT.

Hcy levels were measured before the intervention study to compare the effect of the mutant allele (T). For the statistical analysis, Hcy level of 1 participant with 677CC MTHFR was excluded as it was considered to be an outlier. The means of Hcy level of each SNP were 10.77, 11.67, and 11.83 umol/L, respectively (Fig. 3). The results demonstrated no significant difference regarding Hcy level between 677CT MTHFR polymorphisms but there were slightly higher Hcy values in those who had T allele.
4. Discussion

PCR- RFLP method can be applied for determining MTHFR C677T polymorphisms. Proven in this study, we could differentiate each genotype clearly by looking at the restriction patterns. For genotyping single nucleotide polymorphisms (SNPs) based on endonuclease cleavage, this classic method is a relatively simple, inexpensive, and reliable. Therefore, PCR-RFLP is a good method compared to any other available methods such as hybridization, allele-specific PCR, primer extension, oligonucleotide ligation and endonuclease cleavage [16].

The population was considered to be in Hardy-Weinberg proportions, which follow the assumptions of allelic-frequency and genotypic-frequency equilibrium and random mating. As a whole population, the CC genotype had highest proportion (67%) while TT genotype had lowest proportion (4%) which implied that the presence of C allele was more common than the T allele. Compared to the previous study, Suryandari [17] also reported the same distribution of this genotype in Indonesian population. As result, over 110 subjects collected, we could only find 4 required subjects with homozygous T allele. The intervention study then conducted with a disproportionate number as we use 6 subjects for CC and CT genotype which can lead to abnormal distribution.

The Hcy classification depends on the references by Ingrosso et al. [18]. The mean Hcy level of all genotypes in overweight female adults was in the mild status (10-16 µmol/L). Although we were not able to compare it based on the BMI status, but previous study also shown the higher intima-media-thickness (IMT) and flow-mediated dilatation (FMD) on obese girls, compared with the lean girls, which indicates that the correlation of Hcy elevation with IMT and FMD is apparently mediated by BMI. In adults with a history of CVD, elevated Hcy levels (even mild status) were widely considered to be an independent risk factor [9].

As shown in Fig.3, we found that heterozygotes (CT) had slightly higher concentrations of Hcy levels than people with wild-type genotype (CC). Although not significant, this result might be occurred due to deficiency in MTHFR gene and its role for modification of vitamin B12 metabolism.
as mentioned previously. A meta-analysis study reported that people with the TT genotype have approximately 20% higher Hcy levels but could be vary depending on age, sex, BMI, and other environmental factor [20, 21].

In the present study, we found that the supplementation of folate and vitamin B12 for about 4 weeks give significant reduction of Hcy level, while the placebo giving non-significant effect. This result indicated that Hcy level was dependent on the folate and vitamin B12 status and admission of each individual. Limitation in this study was because there was no measurement in both folate and vitamin B12 status on each subject, but some study also suggested that folate and vitamin B12 deficiencies could trigger Hcy elevation [22]. Still, a further investigation about the correlation of MTHFR gene, B-vitamin supplementation, and Hcy levels as biomarker with the relationship to CVD development is needed.

When we stratified the 4-weeks result of intervention study according to the expression of T allele, we found no significant increase in the all genotype. This result implied the lack of association between the presence of T allele and the high Hcy levels in subjects. However, there was a trend that the effect of supplementation given was observed to be the highest in subject with heterozygotes (TT), followed by CT and CC subjects. This trend was supported by the previous study done by Liu et al. [20] that found the Hcy-lowering effect of the folate supplement was significant in subjects with the 677T allele, but not in 677C carriers. Interestingly, Anna et al. [23] also reported the decrease of TT genotype was greater but more significant when treated with supplement. Yet, surprisingly the Hcy level after those vitamin treatments were higher than the initial values, except for the 677CC subjects. However, the non-significance result could be possibly explained by the presence of many kinds of factors that control Hcy, such as other polymorphisms in the MTHFR gene which leads to mild HHcy and homocystinuria (e.g. A1298C, C599T, G482A, A983G), polymorphisms other genes that contributes in Hcy metabolism (e.g. A2756G in MTR, A66G in MTRR, 680bp insertion at exon 8 CBS gene); renal status, and folate and vitamin B12 status.

Several limitations exist in this study were the number of population, the length of intervention, and the lack of biochemical parameter measurement. As we focused more on the prevention stage of CVD, which targeting on population with moderate characteristics (overweight BMI, adult age with no severe CVD incidences), this could be the possible explanation why no significance found in this study. Moreover, this result can be used as the preliminary study for further research.

5. Conclusion

In summary, Hcy levels are influenced by two critical functions, the genetic factor which may not be changed, and nutritional factor. Subjects with overweight BMI status have mild-range status of Hcy levels which tended to be higher on subjects who had T allele. Admission of folate and vitamin B12 during 4-weeks was proven to lower the level of Hcy level with subject possessed the 677TT MTHFR genotype as the the most responsive ones. Moreover, although there are several limitations, this research could be used as the preliminary study focusing of Hcy elevation.
on overweight female adult in Indonesian population towards CVD prevention.

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Conflict of Interest

The authors report no conflicts of interest

References