Original Article

Serum Folate and Low-Density Lipoprotein Particle Size

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***Aim*: Nutritional parameters, such as B-vitamins, have not been studied for an association with low-density lipoprotein (LDL) particle size. The present study explored whether serum vitamin levels, including folate and vitamin B-12, could be associated with LDL particle size.**
***Methods*: Using a randomly selected population of 255 hospital workers, we collected detailed lipid profiles, including triglyceride (TG), high-density lipoprotein (HDL) cholesterol, and LDL particle sizes. The peak particle size of LDL was measured by density gradient ultracentrifugation and a pore gradient lipoprotein system. Serum folate and vitamin B-12 levels were measured about 1 year later and analyzed. Carotid intima-media thickness (IMT) and hepatic steatosis were diagnosed ultrasonographically, and metabolic syndrome was diagnosed using ATP Ⅲ criteria.**
***Results*: LDL peak particle size was significantly correlated with carotid mean IMT ($r=-0.16$, $p=0.010$). Serum folate levels were significantly and positively correlated with HDL cholesterol and negatively with TG, although the latter showed borderline significance. With increasing serum folate levels, the LDL peak particle size showed a gradual independent increase, even when adjusted for age, sex, hepatic steatosis, metabolic syndrome, and the TG/HDL cholesterol ratio.**
***Conclusion*: Folate may act to enhance LDL particle size. Future clinical and research work should include a study of the safe application and manipulation of folate levels in order to control LDL particle size.**

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Key words; Folate, LDL particle size, TG/HDL cholesterol ratio, Atherosclerosis

Introduction

Atherosclerosis and its associated vascular events, including cardiovascular disease (CVD), stroke, and peripheral arterial disease (PAD), have become a leading cause of disability and mortality in modern society[1], both in developed and developing countries[2]. A lifestyle characterized by a lack of physical activity and moderate to high intake of calories seems to be one of the most important causes of the rapidly increasing prevalence of metabolic syndrome[3], dyslipidemia[4], and eventually atherothrombotic diseases[2].

A sedentary lifestyle causes dyslipidemia; atherogenic dyslipidemia consists of high triglyceride (TG), low high-density lipoprotein (HDL) cholesterol, and smaller low-density lipoprotein (LDL) particle size[5]. Small, dense LDL has been reported to be associated with hepatic steatosis[6], metabolic syndrome[7], PAD[8], and CVD[9]. LDL particle size was reported to be influenced by lipid-lowering agents[10]. The most significant known determinants of LDL particle size are TG and HDL cholesterol[11], and LDL particle size tends not to show independent associations with metabolic parameters and diseases if adjusted further for

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TG and HDL cholesterol or its ratio, an independent predictor of subsequent CVD[12].

As for LDL particle size, previous studies have focused on significant differences in LDL particle size according to the presence of vascular diseases[7-9, 13]; however, nutritional parameters such as B-vitamins, have not been studied for an association with LDL particle size, although LDL particle size is known to be influenced by many factors, from genetics to environmental interaction[14]. In the present study, levels of B-vitamins, including serum folate and vitamin B-12, were measured and studied for an association with LDL particle size using a cohort sample of randomly selected hospital workers.

Subjects and Methods

Study Population

The present cross-sectional study was designed and executed to reveal the relations between metabolic parameters and carotid atherosclerosis. A detailed explanation of the design was reported previously[6, 15]. In brief, according to the age- and sex-stratified random cluster sampling technique, 334 hospital workers were randomly selected and among them, 135 men and 158 women (response 87.7%) participated in the survey. All participants gave their written informed consent to participate in this study, and the study was approved by the appropriate research ethics committee.

Data Collection and Measurements

The administered questionnaire was designed to determine the prior history of CVD, type 2 DM, hypertension, and medication usage (including statins and oral contraceptives). Information regarding the alcohol-drinking status, as estimated by the frequency, duration, amount and kind of liquor consumed, was obtained, and the mean ethanol intake per day was calculated. Smoking status was classified into three categories: current smokers, ex-smokers, and non-smokers. A work index to quantify physical activity was investigated using Baecke's Habitual Activity Questionnaire[16], representing higher index scores as higher levels of physical activity at work, as reported previously[15]. Waist circumference was measured, with the subject standing and wearing no underwear, at the level midway between the lower rib margin and the iliac crest. Body mass index (BMI) was calculated by a computer as weight divided by height squared (kg/m^2).

Abdominal Ultrasonography and Definition of Hepatic Steatosis

All abdominal ultrasonographic scans were performed by one radiologist (Y.K.K.) who was blind to the patients' history and laboratory results. Fatty liver measurements were made using a 3.5-MHz convex probe (Sequoia; Siemens Medical Solutions, Mountain View, CA) in all subjects. Hepatic steatosis was diagnosed by characteristic echo-patterns according to conventional criteria (i.e., evidence of a diffuse increase in echogenicity of the liver as compared with that of the kidney)[17]. Repeated measurements of the same subjects (28 men and 25 women) gave coefficients of variation (CV) of $<1\%$.

Definition of Metabolic Syndrome

Metabolic syndrome was identified by the presence of three or more of the following five components, according to the modified criteria of the Third Adults Treatment Panel (modified ATP-Ⅲ), with waist cutoffs appropriate for an Asian population[18]: 1) abdominal obesity (waist circumference $\geq$90 cm for men and $\geq$80 cm for women); 2) high blood pressure (BP) ($\geq$130/85 mmHg or use of antihypertensive medication); 3) high triglyceride (TG) ($\geq$1.7 mmol/L or 150 mg/dL); 4) low HDL cholesterol ($<$1.03 mmol/L or 40 mg/dL for men and $<$1.3 mmol/L or 50 mg/dL for women); and 5) high fasting glucose ($\geq$5.6 mmol/L or 100 mg/dL).

Carotid Intima-Media Thickness (IMT)

To measure the carotid IMT, we used a higher-frequency 7.0-MHz linear transducer (Sequoia; Siemens Medical Solutions) with compound and harmonic imaging to reduce near-field artifacts. The carotid IMT, a double-line pattern visualized by echotomography on the far wall of both distal common carotid arteries, carotid bulb, and proximal internal carotid arteries, was measured in a region free of plaque, as recommended[19], and their mean values were calculated. Repeated measurements of the same objects (30 subjects) gave a CV $<6.2\%$.

Biochemical Investigations

Blood samples were collected in the morning before breakfast after an overnight fast. Serum biochemistries were assessed with a Hitachi 7600-110 analyzer (Hitachi High-Technologies Corporation, Tokyo, Japan). Most laboratory investigations were described previously in detail[15]. In addition, serum insulin was determined by the electrochemiluminescence immunoassay using Modular Analytics E170 (Roche Diagnostics GmbH, Mannheim, Germany), and insulin resistance was calculated by homeostasis model assessment of the insulin resistance (HOMA-IR) score[20]. Serum TG and glucose were

determined enzymatically (Roche Diagnostics GmbH). HDL cholesterol and LDL cholesterol were measured enzymatically as cholesterol, either after selective disruption of HDL only or using a direct quantitative assay based on an innovative detergent technology, respectively (Daiichi Pure Chemicals Co., Ltd., Japan). Non-HDL cholesterol was calculated as total cholesterol−HDL cholesterol, as recommended[21]. The apolipoprotein A-I (apo A-I) and B (apo B) assays were analyzed by a Roche/Hitachi Modular P Chemistry analyzer that used an immunoturbidimetric assay (Roche Diagnostics GmbH). Serum total homocysteine (tHcy) was measured by fluorescence polarization immunoassay (Axsym; Abbot Laboratories, Abbot Park, IL).

Measurement of LDL Particle Size and Serum Vitamins

The peak particle size of LDL was measured by density gradient ultracentrifugation and a pore gradient lipoprotein system (CBS Scientific, Del Mar, CA) with commercially available non-denaturing 2−16% polyacrylamide gradient gels (Alamo Gels Inc., San Antonio, TX). Standard markers of polystyrene latex beads (36 nm), thyroglobulin (17 nm), apoferritin (12.2 nm) and catalase (10.4 nm) were used to estimate the relative migration rates of each band. The gels were scanned with a GS-800 Calibrated Imaging Densitometer (Bio-Rad Laboratories, Graz, Austria). LDL particle size was calculated with reference to the relative migration value of the standards[22]. According to the LDL peak particle size, pattern B was designated if the particle size was <25.5 nm and otherwise (≥ 25.5 nm), designated as pattern A[23]. For a quantitative subfraction analysis, large, buoyant LDL I (if LDL peak particle size was 27.2−28.5 nm) and small, dense LDL Ⅲ (24.2−25.5 nm) were classified and used for the present study.

To conduct the present study, fasting blood samples were collected about one year later from the same participants (follow-up interval, days, mean ± SD, 321.5 ± 23.5), and serum concentrations of vitamin B-12 and folate were measured by the electrochemiluminescence immunoassay using Elecsys 2010 (Roche Diagnostics GmbH).

Statistical Analysis

Stepwise linear regression analysis for the LDL peak particle size was performed and evaluated for its model fitness. All subsequent analyses were performed according to quartiles of serum folate levels. Analysis of variance (ANOVA) and Student's *t*-test were used to determine statistical differences in continuous variables, and a chi-square test assessed trends in categorical variables. For LDL particle sizes, mean values and 95% confidence intervals (CI) according to the quartiles of serum folate, were analyzed, and for pattern B, a population pyramid showing numbers with 95% CIs was analyzed. A scatter plot of LDL peak particle size and carotid mean IMT is shown to demonstrate their association. A general linear model was used to evaluate the linear relationship between the adjusted mean values of LDL peak particle size and folate level categories (quartiles). Bonferroni tests were applied to correct for multiple comparisons. Interaction terms such as the four categories x variables (i.e., sex) were created, and their significances were assessed. All statistical analyses were conducted using SPSS software version 16.0 (SPSS, Chicago, TX).

Results

Among the 293 participants, 34 (25 women and 13 men, 13.0%) subjects who had left their positions at the hospital during the intervening period did not participate in the follow-up. Another four subjects who did not undergo abdominal ultrasonographic examinations at the first survey were excluded from the present study. Data from 122 men and 133 women (87.0%) were included in the final analysis. The participants were not different from non-participants for age (43.5 ± 6.7 vs. 46.0 ± 10.0, $p=0.164$) and sex (percentage of women; 52.1 vs. 67.6%, $p=0.088$).

With stepwise linear regression analysis, TG, HDL cholesterol, age, serum folate, HOMA-IR, and the apo B/A-I ratio were defined to be associated with LDL peak particle size. The adjusted R^2 of the model with TG and HDL cholesterol was 0.414 and, in the final model, with all variables described above, the model fitness reached 0.477.

Age, BMI, work index, and HOMA-IR were not statistically different according to the quartiles of serum folate, as shown in **Table 1**, but the proportion of women was highest in the third quartile and the smokers in the first quartile. Plasma total homocysteine (tHcy) was significantly decreased and vitamin B-12 was increased according to the quartiles, respectively; however, the proportions of moderate to heavy alcohol drinkers (more than 20 g/d), hepatic steatosis, and metabolic syndrome were not statistically different. About 66 subjects ($n=66$, 25.9%) were defined as having dyslipidemia, and LDL peak particle size was significantly lower in those with dyslipidemia than others (27.0 ± 1.1 vs. 27.5 ± 1.2 nm, respectively, $p=0.006$). Among the subjects with dyslipidemia, 11

Table 1. Characteristics of subjects according to quartiles of serum folate levels

Characteristics	Quartiles of serum folate levels, ng/mL				*p*-value
	1st, <6.43	2nd, 6.43–8.04	3rd, 8.05–10.3	4th, ≥10.32	
Number	62	66	62	65	
Age, y	42.8±5.5	43.0±6.3	43.0±7.6	45.0±7.3	0.190
Women, %	25.8	51.5	66.1	64.6	<0.001
Smoking, ex- and current, %	56.5	43.9	26.2	26.2	<0.001
Alcohol consumed ≥20g/d, %	25.8	28.8	24.2	20.0	0.355
Body mass index (BMI), kg/m^2	23.4±2.6	23.1±2.5	22.9±2.2	23.8±3.1	0.234
Baecke's work index	2.6±0.6	2.5±0.5	2.8±0.7	2.7±0.7	0.122
Plasma total homocysteine, μmol/L	8.8±4.2	7.0±1.6*	6.5±1.5*	6.2±1.4*	<0.001
Vitamin B12, pg/mL	639.7±255.3	708.6±224.2	714.8±157.0	864.0±311.0*†‡	<0.001
HOMA-IR	1.7±2.3	1.4±1.1	1.4±0.8	1.4±1.0	0.622
Hepatic steatosis, %	45.2	41.5	47.5	52.3	0.323
Metabolic syndrome (ATP Ⅲ), %	22.6	13.6	16.1	23.1	0.827

Values are the mean±SD unless noted otherwise. HOMA-IR; homeostasis model assessment-insulin resistance, ATP; adult treatment panel.
p values by analysis of variance (ANOVA) or chi-square test for trend as appropriately.
*$p<0.001$; Compared with the 1st quartile of serum folate (Bonferroni comparisons).
†$p<0.01$; Compared to the 2nd quartile of serum folate (Bonferroni comparisons).
‡$p<0.01$; Compared to the 3rd quartile of serum folate (Bonferroni comparisons).

Table 2. Lipid profiles of the subjects according to the quartiles of serum folate levels

Lipid profiles	Quartiles of serum folate levels, ng/mL				*p*-value
	1st, <6.43	2nd, 6.43–8.04	3rd, 8.05–10.3	4th, ≥10.32	
HDL cholesterol, mmol/L	1.2±0.3	1.3±0.3*	1.3±0.3	1.3±0.3*	0.005
TG, mmol/L	1.6±0.8	1.3±0.9	1.4±0.8	1.4±0.8	0.147
TG/HDL cholesterol ratio	1.5±1.0	1.1±1.1	1.2±0.9	1.2±0.8	0.062
Total cholesterol, mmol/L	4.7±0.8	4.6±0.9	4.7±0.7	4.8±0.8	0.601
LDL cholesterol, mmol/L	2.9±0.7	2.8±0.7	2.9±0.6	2.9±0.7	0.923
Non-HDL cholesterol, mmol/L	3.5±0.7	3.3±0.8	3.5±0.7	3.5±0.9	0.418
Lipoprotein(a), mmol/L	0.7±0.6	0.7±0.5	0.8±0.7	0.7±0.6	0.788
Apo B, g/L	0.83±0.19	0.81±0.22	0.82±0.21	0.83±0.23	0.964
Apo A-I, g/L	1.24±0.38	1.23±0.25	1.23±0.26	1.28±0.26	0.663
Apo B/A-I ratio	0.71±0.23	0.67±0.20	0.70±0.23	0.67±0.23	0.715

p values by ANOVA. HDL; high-density lipoprotein, TG; triglyceride, LDL; low-density lipoprotein, apo B; apolipoprotein B, apo A-I; apolipoprotein A-I.
*$p<0.05$; Compared with the 1st quartile of serum folate (Bonferroni comparisons).

(16.7%) were taking statins, but there was no significant difference in LDL peak particle size according to statins (26.7±0.9 vs. 27.1±1.1 nm, subjects taking statins vs. others, respectively, $p=0.345$). Ten women (7.5%) were taking oral contraceptives, but there was no significant difference in LDL particle size according to this medication ($p=0.548$). The work index showed a positive correlation with LDL peak particle size with borderline significance ($\beta=0.11$, $p=0.092$), and showed significant correlations with percentages of LDL Ⅰ and LDL Ⅲ ($\beta=0.14$ and -0.17, $p=0.022$ and 0.007, respectively).

Various lipid profiles are presented in **Table 2**. Among them, HDL cholesterol showed a significant increase according to the increase of serum folate levels and the TG/HDL cholesterol ratio showed borderline significance. All other lipid profiles, including TG, LDL cholesterol, lipoprotein(a), and the apo B/A-I ratio, were not significantly different across the quartiles.

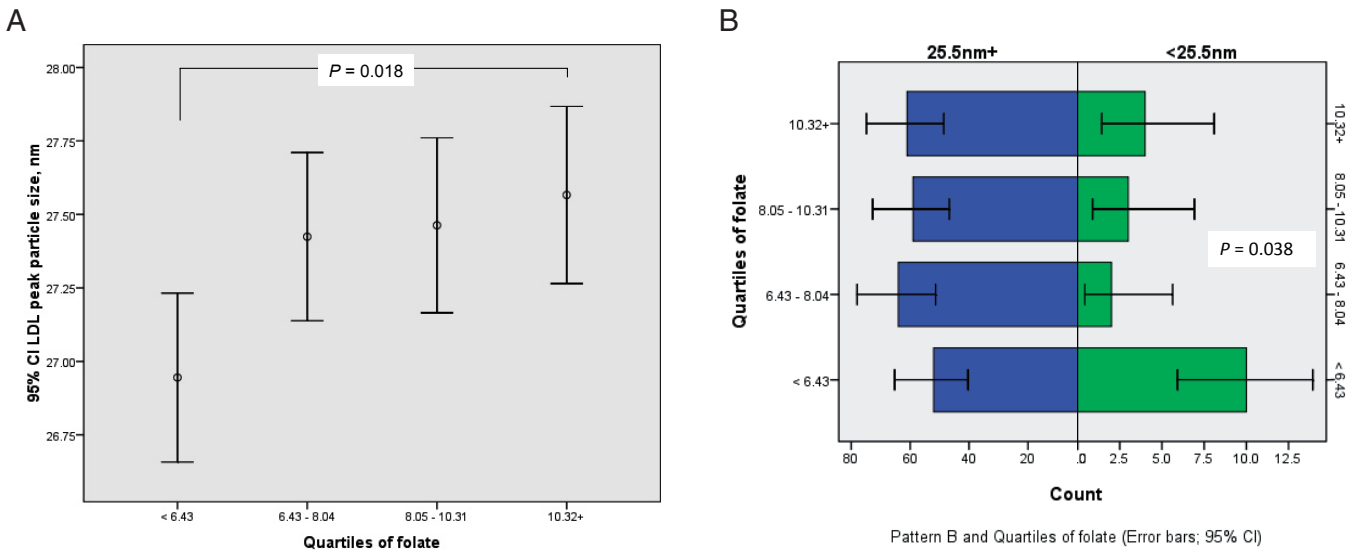

Fig. 1. LDL particle sizes and pattern B (<25.5 nm) according to the quartiles of serum folate levels (ng/mL). Relations between quartiles of folate levels and LDL peak particle size (p=0.018, 1A). Pattern B was significantly higher in the lowest quartile of folate (16.1%, p=0.038, 1B). Notice that the scales for the count number were different in Fig. 1B according to Pattern A or B.

LDL peak particle size increased significantly with the increase of folate levels, as depicted in **Fig. 1A** (p=0.018). Pattern B (<25.5 nm, 7.5% overall) was most prevalent in the lowest quartile of folate levels (n=10, 52.6%), as in **Fig. 1B** (p=0.038). A scatter plot of the LDL peak particle size and carotid mean IMT showed a significant negative correlation (r=−0.16, p=0.010), as depicted in **Fig. 2**. As for effect modifiers for the association presented in **Fig. 1A**, neither sex showed significant interaction terms, nor other factors, such as alcohol consumption and nonalcoholic fatty liver disease (NAFLD); (all p for interaction >0.1).

Adjusted mean values of LDL peak particle size according to the quartiles of folate levels were significantly increased, as shown in **Table 3**, and the association between LDL peak particle size and folate levels was independent, even when adjusted for age, sex, hepatic steatosis, metabolic syndrome, HOMA-IR, and the TG/HDL cholesterol ratio.

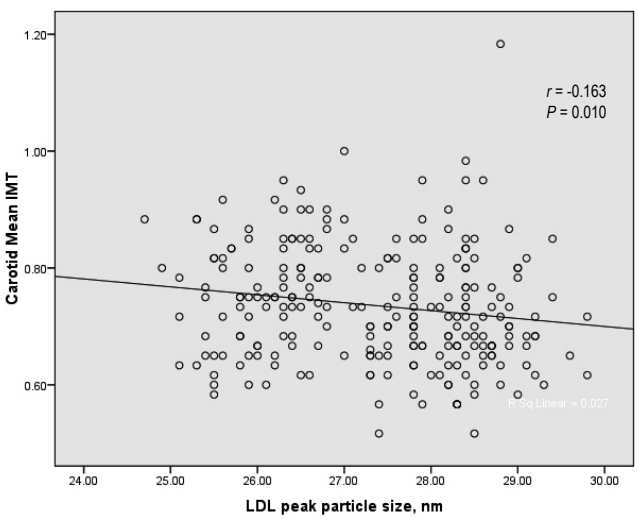

Fig. 2. A scatter plot of LDL peak particle size and mean values of carotid intima-media thickness (IMT), showing a significant correlation coefficient of r=−0.16, p=0.010. Note that a significant association was observed even when the LDL peak particle size was larger than 25 nm.

Discussion

In the present study, LDL particle sizes and the proportion of pattern B showed significant associations with serum folate levels. LDL peak particle size, which was proven to be significantly correlated with both the work index and carotid mean IMT, was independently associated with serum folate levels, even when adjusted for metabolic syndrome and TG/HDL cholesterol ratio, which are known as the most highly correlated factors with LDL peak particle size[11]. We believe that this is the first report to show an independent association between serum folate levels and LDL peak particle size.

As high amounts of serum TG are released as

Table 3. Adjusted mean (±SE) values of LDL peak particle size according to the quartiles of serum folate levels

Models	LDL peak particle size (nm) according to the quartiles of folate levels, ng/mL				*p*-value	
	1st, <6.43	2nd, 6.43–8.04	3rd, 8.05–10.3	4th, ≥10.32	(Between groups)	(Linear trend)
Crude	26.9±0.2	27.4±0.1	27.5±0.1*	27.6±0.1**	0.007	0.005
Model 1	27.0±0.1	27.4±0.1	27.4±0.1	27.6±0.1*	0.028	0.018
Model 2	26.9±0.1	27.4±0.1	27.4±0.1	27.7±0.1**	0.003	0.001
Model 3	27.0±0.1	27.3±0.1	27.4±0.1	27.6±0.1**	0.012	0.004

Model 1 included age, sex, smoking, alcohol consumption (≥20g/d), tHcy, vitamin B-12, HOMA-IR, and work index; Model 2 included variables in model 1 plus hepatic steatosis; Model 3 included variables in model 2 plus BMI, LDL cholesterol, metabolic syndrome, and TG/HDL cholesterol ratio.
*$p<0.05$, **$p<0.01$; Compared with the 1st quartile of serum folate (Bonferroni adjustment for multiple comparisons).

very low density lipoprotein (VLDL) from the liver, TG-rich HDL cholesterol formed by the interaction with cholesterol ester transfer protein (CETP) undergoes hydrolysis by lipoprotein lipase (LPL), and is eventually degraded by the kidney[24]. TG-rich lipoproteins (TRLs) deplete TG by lipolysis, thus giving rise to small, dense LDL particles[25]. LDL particle size, *in vivo*, is closely related with serum levels of TG and HDL cholesterol, so the independent association between LDL peak particle size and serum folate levels, even when adjusted for the TG/HDL cholesterol ratio, merits attention.

Folate is used to reduce plasma tHcy[26], which was independently associated with increased cerebral arterial resistance[27] and internal carotid arterial occlusion[28], as the present authors reported previously. Folate also had modulating activity on LPL[29], an important determinant of HDL-cholesterol[30]. In addition, folate appears to interact with apolipoprotein E (apoE), a ligand for LDL receptors, as for both activity and apoE DNA methylation[31]. The interaction between folate and apoE appears to affect serum lipid levels, because after a classical degradation of LDL, apoE, which is involved in the lipolysis of TRLs, could be mobilized by HDL or apo A-I, and recycled back to plasma[32]. APOE polymorphism also had a significant effect on lipid levels and there were APOE-environment interactions[33]. The exact role of folate in apoE and APOE, however, should be elucidated further.

The present study has important limitations. First, the blood for serum folate measurements was obtained one year later than the blood for LDL particle size determinations, so there could be a causal direction from LDL particle size to vitamins. Although the cross-sectional relationship and the direction between them were unknown, the adjusted models, including all possible confounders, as in **Table 3** seemed to potentially substantiate their true relations. Second, the present study did not survey for information about food or nutrient consumption, such as folate and niacin. Thus, we cannot determine whether the serum folate levels were decided by food intake only or modified by additional nutrient supplements. Third, compared to a previous report[34], the percentage of pattern B was very small in this sample of Korean hospital workers. Another study using representative data is necessary in order to compare the exact proportion of pattern B in general Korean populations. Fourth, the population in the present study was composed largely of middle-aged persons more than 40 years old, so the present study could not reveal whether the association might be modified by age, which was suggested originally by previous researchers[14].

Summary

The present study showed an independent association between serum folate levels and LDL peak particle size, even when adjusting for the TG/HDL cholesterol ratio. Folate may act on the determination process of LDL particle size. The physiologic mechanism for the independent association between serum folate levels and LDL peak particle size should be studied further, but folate is a safe and effective compound to reduce the concentration of tHcy. Thus, we suggest that targeted trials to quantify the effects of folate on LDL particle size should be safely implemented for future clinical and research study.

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Authors Contributions:

DSK and YKK participated in data collection and reviewed the manuscript. HKP and YIC conceived the study and reviewed the manuscript. SKJ participated in data collection, conceived the study, drafted, and reviewed the manuscript. All authors read and approved the final manuscript.

Conflicts of Interests: None declared.

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