Associations of Homocysteine with B Vitamins and Zinc in Serum Levels of Patients with Type 2 Diabetes Mellitus: A Cross-Sectional Study

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Summary The association of homocysteine metabolism-related nutrients along with renal function to homocysteine levels is not well known in patients with type 2 diabetes mellitus (T2DM). We investigated the relevance of kidney function, albuminuria, and nutritional factors to serum homocysteine in T2DM patients. This cross-sectional study enrolled 149 T2DM patients (96 men and 53 postmenopausal women), and patient characteristics and laboratory data including kidney-related data [glomerular filtration rate (eGFR), urinary albumin excretion (UACR), uric acid] and metabolism parameters (hemoglobin A1c and lipids) were collected from the medical record and serum levels of vitamin B12, folic acid, zinc, homocysteine and UACR were also acquired. In total subjects, serum levels of homocysteine, vitamin B12, and folic acid were within reference intervals, but zinc levels were close to lower limits of its reference interval. A multivariate-adjusted analysis showed that gender (β=−0.259, p<0.001), uric acid (β=0.267, p<0.001), eGFR (β=−0.188, p=0.001), log UACR (β=0.190, p=0.002), log folic acid (β=−0.259, p<0.001), log vitamin B12 (β=−0.224, p<0.001) and zinc (β=−0.169, p=0.006) were correlated to log homocysteine. In multiple regression analysis by gender, these correlations were found similarly in men, but neither log folic acid nor zinc showed correlations with log homocysteine in women. The present study suggests that renal function parameters and the certain nutritional factors have a possible influence on serum homocysteine, in T2DM patients including diabetes kidney disease.

Key Words homocysteine, folic acid, vitamin B12, zinc, type 2 diabetes mellitus, diabetic kidney disease, renal function

Hyperhomocysteinemia is considered a risk factor for cardiovascular diseases (CVDs), including coronary heart disease (CHD) and stroke, as previously reported (1, 2). Hyperhomocysteinemia, a non-classical CVD risk factor as well as microalbuminuria in chronic kidney disease (CKD), is found in end-stage renal disease (3, 4). The mechanisms by which serum homocysteine increases in CKD patients are not well defined, and little is known about the influence of increased homocysteine on kidney tissues. Higher levels of serum homocysteine are found in patients with type 2 diabetes mellitus (T2DM) than in subjects without diabetes, and serum homocysteine concentrations are higher in diabetic patients with CKD than in those without CKD (5, 6). A meta-analysis paper shows that the high level of plasma homocysteine is associated with both the risk and severity of nephropathy in patients with T2DM (7). However, the relevance of nutritional factors to serum homocysteine under the influence of renal function and urinary albumin excretion remains undefined in T2DM patients.

An increase in serum concentration of homocysteine, an intermediate metabolite of methionine, is known to be attributable to the high percentage of methionine in dietary protein and lack of B vitamins related to the metabolism of homocysteine (8, 9). Several nutrients, including folic acid and zinc, are involved in the metabolic process of re-methylation of homocysteine to methionine. Zinc is a constituent of betaine-homocyste-

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ine methyltransferase, another re-methylation enzyme, and previous papers have reported that serum zinc concentrations are low in patients with T2DM and CKD (10–13). Nevertheless, the relevance of homocysteine metabolism-related nutrients, especially vitamin B12, folic acid, and zinc, under the influence of renal function and urinary albumin excretion to serum homocysteine levels is not well known in patients with T2DM.

Consequently, the purpose of our study was to investigate the relevance of homocysteine metabolism-related nutritional factors, especially vitamin B12, folic acid, and zinc, under the influence of renal function and urinary albumin excretion to serum homocysteine in patients with T2DM.

MATERIALS AND METHODS

Study design and patient selection. This study was based on a retrospectively cross-sectional design, and the subjects were T2DM patients who visited the outpatient clinic of internal medicine of diabetes, metabolism, and endocrinology at the Jikei University Kashiwa Hospital from August 2018 to May 2020. The excluded criteria in the present study were subjects with poor glycemic control [glycohemoglobin A1c (HbA1c) levels >9.0%), secondary diabetes, endocrine diseases, gastrointestinal disorders, steroid therapy, estimated glomerular filtration rate (eGFR) under 30 mL/min/1.73 m². Not only serum lipids but also serum homocysteine concentrations are known to be affected by female hormones so that the present study excluded premenopausal women (14).

This study conformed to the Declaration of Helsinki and was approved by the Ethics Committee of the Jikei University School of Medicine (approval number: 30-010), and a waiver for informed consent was approved with the opt-out consent.

Data collection and measurements. Patient characteristics [age, gender, body mass index (BMI), blood pressures, status of medication, habits of alcohol and smoking] and laboratory data including glucose, lipids, and uric acid (UA) of fasting samples were collected from electronic medical records. The subjects who had a habit of drinking alcohol more than 60 g/wk were defined as a regular drinker, and daily smokers and occasionally smokers with smoking for more than 6 mo were defined as a current smoker.

Serum levels of low-density lipoprotein (LDL) cholesterol (LDL-C) was determined by Friedewald formula [LDL-C=total cholesterol (TC)−high density lipoprotein (HDL) cholesterol (HDL-C)−triglyceride (TG)/5] (15). TC and TG were measured by conventional methods with Determiner LTCC and TG (Minaris Medical, Hitachi, Japan). HDL-C was measured by a direct method with Metabolead HDL-C (Minaris Medical). Hba1c was measured by high-performance liquid chromatography with glycohemoglobin analyzer HA-8190V (Arkray, Japan). Creatinine and UA were measured by routine enzymatic methods with L type Wako CRE M and L type Wako UA M (FUJIFILM Wako Pure Chemical Corporation, Japan), respectively. Serum levels of vitamin B12, folic acid, zinc, total homocysteine, and urinary albumin excretion [urine albumin-to-creatinine (Cr) ratio (UACR, mg/gCr)] were additionally measured by using residual samples when any one of these was missing in laboratory data. Serum levels of vitamin B12 and folic acid were measured by chemiluminescent enzyme immunoassay (CLEIA) using Access folic acid or Access B12 (Beckman Coulter Japan), respectively. Serum levels of zinc and total homocysteine were measured by colorimetry assay with espa Zn II (NIPRO, Japan) and by high-performance liquid chromatography using YMC-Triart C18 (YMC, Japan), respectively. UACR was measured by immuno-nephelometry using Autokit Micro Albumin (FUJIFILM Wako Pure Chemical Corporation). In addition, eGFR (mL/min/1.73 m²) was calculated using the following formula (16): 194×serum creatinine (mg/dL)−1.094×age (y)−0.287 for men and 0.739×this calculated eGFR for women. The diagnosis of diabetic kidney disease (DKD) was given by the classification of Diabetic Nephropathy 2014 (17).

Statistical analysis. All statistical analyses were performed using a statistical package for social science (SPSS, version 25.0 (IBM, Tokyo, Japan). Assuming an α level of 0.05, 80% power and 0.15 effect size, the required number of patients for each group to observe a homocysteine difference was determined 127 in total by using G*Power version 3.1.9.7 (Franz Faul, Kiel University, Germany) (18).

The data were expressed as number and frequency (%) or mean±standard deviation (SD). Categorical variables were assessed by Chi-square test or Fisher’s exact test, and stratified analysis to continuous variables between the two groups categorized by gender were performed using unpaired t-test or Mann-Whitney U test. For regression analysis, UACR, TG, homocysteine, vitamin B12 and folic acid were not shown to normal distribution so that these variables were converted to logarithmetic values. Simple correlations between log homocysteine and continuous variables were evaluated by Pearson correlation analysis, and hierarchical multiple regression analyses were performed for log homocysteine as an objective variable as follows: Model 1, forced entry regression analysis was performed with universal confounding factors (age, gender, BMI) and other explanatory variables (eGFR, log UACR, and Hba1c); Model 2, forced regression analysis was performed with UA, log folic acid, log vitamin B12, and zinc as explanatory variables in addition to those in Model 1. All p values of <0.05 were considered significant.

RESULTS

Characteristics of study subjects

Physical and biochemical data. A total of 149 patients (96 men and 53 postmenopausal women) were enrolled in this study. Table 1 shows the characteristics of study subjects. In total subjects, mean values of age, BMI, and Hba1c were 68±11 y old, 25.8±4.2 kg/m², and 7.5±1.0%, respectively. In kidney function-related indicators, mean values of eGFR and UACR were 61.4±19.4 mL/min/1.73 m² and 441.2±926.4 mg/gCr, respectively. The T2DM patients with CKD (<60 of eGFR)
were found in 52 men (54.2%) and 14 women (26.4%), and microalbuminuria (UA-Cr ≥ 30) were found in 66 men (68.8%) and 23 women (43.4%). On the other hand, 8.7% (n = 13) and 18.8% (n = 28) of total subjects met CKD criteria for normal albuminuria and macroalbuminuria, respectively.

Serum levels of homocysteine, vitamin B12, and folic acid were within reference intervals, but zinc levels were close to lower limits of its reference interval (>80 μg/dL). Blood pressures, creatinine, UA, UACR, and homocysteine were higher in men than in women. However, eGFR, TC, and LDL-C were lower in men than in women.

Table 2. Status of medication, habits of drinking and smoking

|                        | Total (n=149) | Men (n=96) | Women (n=53) | p-value |
|------------------------|--------------|------------|--------------|---------|
| Anti-diabetic drugs    |              |            |              |         |
| (n, %)                 |              |            |              |         |
| Metformin (n, %)       | 147 (98.7)   | 96 (100.0) | 51 (96.2)    | 0.125   |
| Anti-hypertensive drugs(n, %) | 62 (41.6) | 37 (38.5) | 25 (47.2) | 0.306 |
| Lipid-lowering drugs   | 85 (57.0)    | 52 (54.2)  | 33 (62.3)    | 0.339   |
| Acid lowering drugs**  | 28 (18.8)    | 25 (26.0)  | 3 (5.7)      | 0.001   |
| Regular drinker**      | 24 (16.1)    | 23 (24.0)  | 1 (1.9)      | <0.001  |
| Current smoker         | 28 (18.8)    | 22 (22.9)  | 6 (11.3)     | 0.083   |

Table 1. Characteristics of study subjects.

Data are expressed as mean ± standard deviation (SD). Mann-Whitney U test (*) or unpaired t-test were used for statistical analyses about continuous variables.

BMI, body mass index; eGFR, estimated glomerular filtration rate; UACR, urine albumin-to-creatinine ratio; HbA1c, glycohemoglobin A1c; HDL-C, high-density lipoprotein cholesterol; LDL-C, low-density lipoprotein cholesterol.

Data are expressed as number and frequency (%). The chi-square test or Fisher’s exact test (**) were used for statistical analyses about continuous variables.

Significant simple correlations of log homocysteine with the subject characteristics and biochemical data

Significant simple correlations of log homocysteine in total subjects were found positively with UA and log UACR, and inversely with eGFR, log vitamin B12, log folic acid, and zinc. However, age, BMI, blood pressures, and HbA1c were not correlated with log homocysteine in total subjects.
Multiple regression analysis about log homocysteine, an objective variable, were performed using data of eGFR, HbA1c, UA, log UACR, log vitamin B12, log folic acid, and zinc as explanatory variables with universal confounding factors (gender, age, and BMI). The multiple regression analysis in the Model 1 showed the significant correlations of eGFR, log UACR and gender with log homocysteine (Table 4). In the Model 2, independent correlations of UA (β=0.267, p<0.001), gender (β=−0.259, p<0.001), log UACR (β=0.190, p=0.002), eGFR (β=−0.188, p=0.001), log folic acid (β=−0.259, p<0.001), log vitamin B12 (β=−0.224, p<0.001), and Zn (β=−0.169, p=0.006) were significantly found with log homocysteine (Table 4). Moreover, we performed a multivariate regression analysis by the difference of glycemic control status (HbA1c levels <7% or ≥7%). As a result, the serum UA was an independent explanatory factor for serum homocysteine.

The gender difference was independently correlated with log homocysteine so that subsequently multiple regression analysis was performed by gender (Table 5). In men, log folic acid (β=−0.352, p<0.001), log vitamin B12 (β=−0.194, p=0.021), log UACR (β=0.249, p=0.005) and log eGFR (β=−0.194, p=0.022) significantly correlated with log homocysteine in the Model 2. However, in women, log vitamin B12 (β=−0.407, p=0.002) and eGFR (β=−0.234, p=0.048) but neither log folic acid nor zinc significantly correlated with log homocysteine.

**DISCUSSION**

The present study showed the significant correlations of renal function parameters and homocysteine metabolism-related nutritional factors with serum homocysteine in T2DM patients including DKD. DKD is a concept that includes not only typical diabetic nephropathy but also diabetes-related renal disease accompanied by a reduction of eGFR without macroalbuminuria (19, 20). In the total subjects (men and postmenopausal women), the multivariate regression analysis showed the significant inverse relevance of not only eGFR but also vitamin B12, folic acid, and zinc to homocysteine, while the significant positive relevance to homocysteine was found in UA and UACR in total subjects. These results are certainly consistent with the evidence of previous papers that reported an increase in serum homocysteine and the essential contribution of B vitamins to homocysteine metabolism in patients with CKD and diabetes (3–5, 21). Previous studies reported that the fortification of not only folic acid but also vitamin B12 certainly reduced serum homocysteine levels (22, 23).

Folic acid has a methyl donor function as a substrate source of 5-methyltetrahydrofolate, and methionine synthase for re-methylation to methionine needs vitamin B12 as a cofactor. As well as methionine synthase, betaine-homocysteine methyl transferase also is one of zinc metalloenzymes. From the results of multiple

### Table 3. Simple correlations between log transformed homocysteine and numerical data of characteristics.

| Characteristics     | r     | p-value |
|---------------------|-------|---------|
| Age                 | 0.102 | 0.217   |
| BMI                 | 0.036 | 0.666   |
| Systolic blood pressure | 0.147 | 0.073   |
| Diastolic blood pressure | 0.047 | 0.566   |
| eGFR                | −0.345| <0.001  |
| Uric acid           | 0.404 | <0.001  |
| log UACR            | 0.369 | <0.001  |
| HbA1c               | 0.058 | 0.484   |
| Total cholesterol   | −0.078| 0.343   |
| HDL-C               | −0.013| 0.871   |
| LDL-C               | −0.094| 0.255   |
| log Triglyceride    | −0.006| 0.938   |
| log Vitamin B12     | −0.250| 0.002   |
| log Folic acid      | −0.378| <0.001  |
| Zinc                | −0.291| <0.001  |

*“r” value indicates Pearson’s rank correlation coefficient. Abbreviations are referred to the Table 1 footnotes.*

### Table 4. Multivariate-adjusted regression between log transformed homocysteine and explanatory factors in total subjects.

| Extracted explanatory variables | β     | p-value |
|---------------------------------|-------|---------|
| Model 1                         |       |         |
| Age                             | 0.040 | 0.553   |
| Gender                          | −0.378| <0.001  |
| BMI                             | 0.011 | 0.872   |
| eGFR                            | −0.254| <0.001  |
| log UACR                        | 0.246 | 0.001   |
| HbA1c                           | 0.044 | 0.527   |
| R²                              | 0.336 | <0.001  |
| Model 2                         |       |         |
| Age                             | 0.071 | 0.254   |
| Gender                          | −0.259| <0.001  |
| BMI                             | 0.026 | 0.645   |
| eGFR                            | −0.188| 0.001   |
| log UACR                        | 0.190 | 0.002   |
| HbA1c                           | 0.057 | 0.330   |
| Uric acid                       | 0.267 | <0.001  |
| log Folic acid                  | −0.259| <0.001  |
| log Vitamin B12                 | −0.224| <0.001  |
| Zinc                            | −0.169| 0.006   |
| R²                              | 0.549 | <0.001  |

Model 1: The multivariate analysis was performed by forced entry regression method, the objective variable was log homocysteine, and explanatory variables were the universal confounding factors (age, gender, BMI), eGFR, log UACR, and HbA1c. Abbreviations are referred to the Table 1 footnotes.

Model 2: The multivariate analysis was conducted by forced entry regression method, the explanatory variables were uric acid, log folic acid, log vitamin B12, and zinc in addition to the explanatory variables of Model 1. β means adjusted R-squared, and R² shows multiple coefficients of determination.
Therefore, the clinical relevance of zinc as one of important nutrients to homocysteine metabolism is considered a remaining issue in patients with T2DM.

On the other hand, metformin therapy has been reported to decrease serum levels of vitamin B12, and long-term use of metformin is associated with vitamin B12 deficiency. However, metformin does not affect circulating concentrations of vitamin B12 and folic acid at least during the first 6 mo after initiating (30, 31). In our study, over half of total subjects were metformin users, but the average of serum vitamin B12 concentrations was within normal ranges. Consequently, we thought that the metformin therapy did not influence serum vitamin B12 concentrations in this study.

In the present study, serum uric acid showed the highest contribution as an explanatory factor to serum homocysteine regardless of glycemic control. The association between uric acid and serum homocysteine can be explained by hydrolysis of S-adenosyl homocysteine leading to the formation of homocysteine and adenosine, because adenosine is degraded to form uric acid as its end-product (32). In the previous studies, the positive correlation between homocysteine and serum uric acid were observed (33–35), our results are consistent with these studies. However, there are also previous papers which reported that no correlations were found between serum homocysteine and serum uric acid in patients with gout or with uric acid-lowering treatment (36, 37). It is known that high uric acid can exert beneficial functions due to its antioxidant properties, sug-

### Table 5. Multivariate-adjusted regression between log transformed homocysteine and relevant factors by gender.

| Extracted explanatory variables | Men (n=96) | Women (n=53) |
|---------------------------------|-----------|-------------|
|                                 | β         | p-value     | β          | p-value     |
| Model 1                         |           |             |            |             |
| Age                             | 0.003     | 0.977       | 0.124      | 0.361       |
| BMI                             | 0.028     | 0.779       | -0.020     | 0.877       |
| eGFR                            | -0.271    | 0.008       | -0.281     | 0.040       |
| log UACR                        | 0.261     | 0.009       | 0.303      | 0.020       |
| HbA1c                           | -0.034    | 0.736       | 0.191      | 0.132       |
| R²                              | 0.105     | <0.001      | 0.213      | <0.001      |
| Model 2                         |           |             |            |             |
| Age                             | 0.069     | 0.777       | 0.092      | 0.462       |
| BMI                             | 0.071     | 0.392       | -0.041     | 0.714       |
| eGFR                            | -0.194    | 0.022       | -0.234     | 0.048       |
| log UACR                        | 0.249     | 0.005       | 0.192      | 0.101       |
| HbA1c                           | -0.014    | 0.566       | 0.171      | 0.134       |
| Uric acid                       | 0.261     | 0.003       | 0.314      | 0.013       |
| log Folic acid                  | -0.352    | <0.001      | -0.142     | 0.286       |
| log Vitamin B12                 | -0.194    | 0.021       | -0.407     | 0.002       |
| Zinc                            | -0.228    | 0.012       | -0.195     | 0.113       |
| R²                              | 0.401     | <0.001      | 0.430      | <0.001      |

Model 1: The multivariate analysis was performed by forced entry regression method, the objective variable was log homocysteine, and explanatory variables were the universal confounding factors (age, BMI), eGFR, log UACR, and HbA1c. Abbreviations are referred to the Table 1 footnotes.

Model 2: The multivariate analysis was conducted by forced regression method, the explanatory variables were uric acid, log folic acid, log vitamin B12, and zinc in addition to the explanatory variables of Model 1. β means adjusted R-squared, and R² shows multiple coefficients of determination.
gesting that homocysteine and uric acid are interactive in redox actions in association with cardiovascular disease risk (2). The relations of serum uric acid and homocysteine need to be confirmed in further studies.

Moreover, UACR also showed a significant correlation to serum homocysteine in total subjects and men, but such a correlation was not seen in women. The previous studies reported the association between elevated serum homocysteine and higher prevalence of albuminuria (34), and hyperhomocysteinemia was associated with increased risk of microalbuminuria in patients with T2DM (6). In our study, the serum homocysteine level was significant higher in men than in women, and the UACR levels were mostly at the levels of classified macroalbuminuria in total subjects and men, but the women’s UACR levels were at the levels of microalbuminuria. These differences in UACR levels might affect the relevance of UACR to homocysteine.

The present study has several limitations to be mentioned. First, this research was a cross-sectional study, which provides no evidence of causal relationship between serum homocysteine and nutritional factors, including folic acid, vitamin B12, and zinc. Second, the dietary intake amounts of B vitamins and zinc were unknown because this retrospective study did not acquire dietary records. Third, the present study investigated the serum homocysteine levels focusing only on nutritional factors related to the re-methylation pathway. However, the metabolism of homocysteine is regulated by the pathway of trans-sulfuration to cystathionine-by-cystathionine β-synthase in an irreversible vitamin B6-dependent reaction in addition to the remethylation pathway (9). Fourth, whether the present study results can be extrapolated to other subjects except for T2DM patients remains to be investigated. Pastore et al. (38) reported no significant correlations between B-vitamins and severity of hyperhomocysteinemia regardless of the CKD stage in T2DM patients. Previous homocysteine-lowering intervention studies in renal failure patients revealed that folic acid has the most powerful homocysteine-lowering effect although the beneficial effects of vitamin supplementation from these clinical trials are not consistent (39). The comparison of nutritional factors in relation to serum homocysteine levels between kidney disease with and without diabetes should be investigated in the future.

In terms of dietary therapy for T2DM patients, the sufficient intake of vitamins and minerals would be recommended, but no clear relevance of the dietary vitamins and minerals to the management of diabetes was reported previously (40). Prospective intervention studies will be needed for the assessment of blood levels of vitamins and minerals in T2DM patients with hyperhomocysteinemia in the future.

CONCLUSIONS

The present study suggests that renal function have a significant influence on serum homocysteine, accompanied by the concurrent relevance of nutritional factors (vitamin B12, folic acid, and zinc), in T2DM patients including DKD. However, the reasons for a gender difference in these nutritional factors affecting serum homocysteine level remain to be cleared.

Authorship

SM, CH and HY designed the concept and methods of this study. KA and KF selected the study subjects. RS and TK collected the blood samples and provided additional biochemical data. SM and CH collected information including drug medication from medical records. SM analyzed and interpreted the patient data and wrote the original draft preparation. SM and HY were mainly in charge of the manuscript preparation, and HY supervised the manuscript. All authors read and approved the final manuscript.

Disclosure of state of COI

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