Homocysteine, cysteine, folate and vitamin B_{12} status in type 2 diabetic patients with chronic kidney disease

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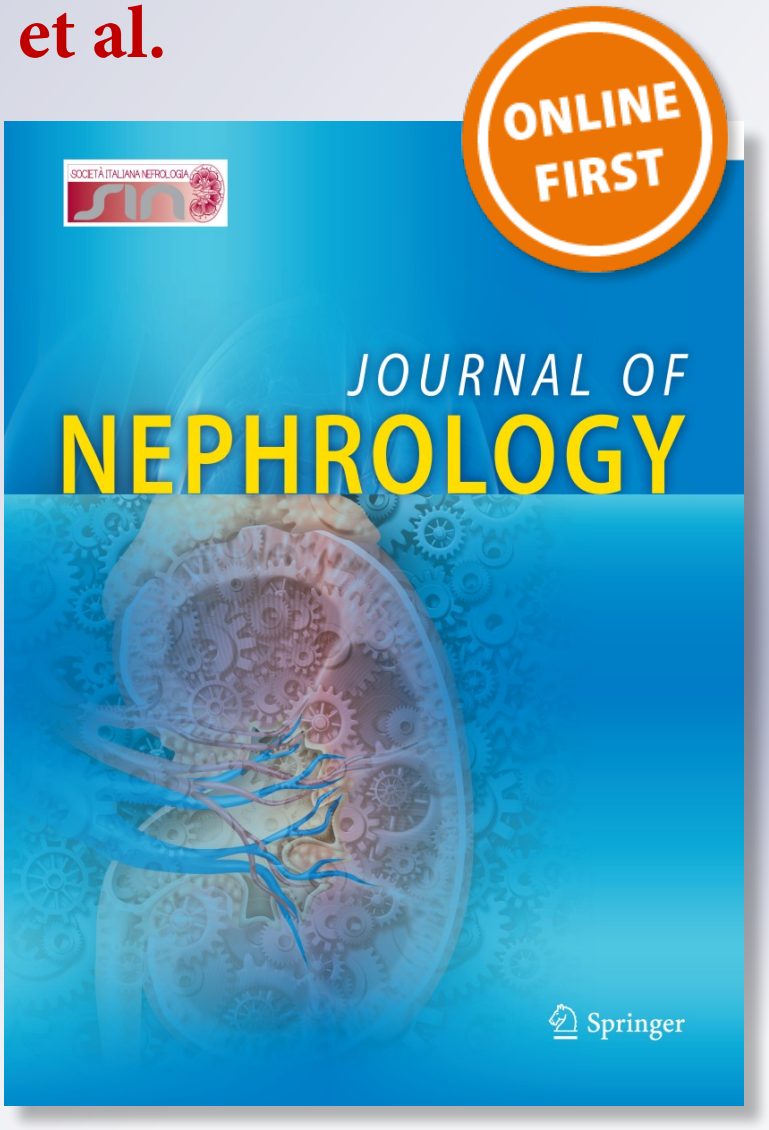
Journal of Nephrology

ISSN 1121-8428

J Nephrol
DOI 10.1007/s40620-014-0126-4



Springer

J Nephrol
DOI 10.1007/s40620-014-0126-4

ORIGINAL ARTICLE

Homocysteine, cysteine, folate and vitamin B_{12} status in type 2 diabetic patients with chronic kidney disease

Anna Pastore · Annalisa Noce · Gianna Di Giovamberardino · Alessandro De Stefano · Cinzia Callà · Rossella Zenobi · Mariarita Dessì · Nicola Di Daniele

Received: 7 April 2014 / Accepted: 20 July 2014

Abstract
Background Hyperhomocysteinemia (hHcy) is a risk factor in the progression of chronic kidney disease (CKD). In type 2 diabetes (T2D), hHcy is strongly associated with increased risk of cardiovascular disease. Vitamin B_{12} and folic acid supplementation have been reported to lower homocysteine (tHcy) levels, but no data on plasma tHcy, cysteine (Cys), folate and vitamin B_{12} levels in T2D-CKD patients are reported.
Procedures tHcy and Cys levels were analyzed in 178 T2D-CKD patients by high performance liquid chromatography (HPLC) with fluorescence detection. In addition, we determined folate and vitamin B_{12} levels using a chemiluminescence method.
Results tHcy and Cys levels were increased in T2D patients, and this rise positively correlated with the CKD stage ($P < 0.001$). Folate levels were comparable to controls at various CKD stages, whereas vitamin B_{12} levels were lower, except at stage IV. We did not find any correlation between B-vitamins and levels of tHcy and Cys, regardless of the CKD stage.
Conclusions This is the first study reporting tHcy, Cys and B-vitamins status in T2D-CKD patients. Although limited to our cohort of 178 patients, our findings could be helpful in clarifying the conflicting literature regarding B-vitamins supplementation. Further studies are necessary before any Hcy-lowering therapy can be safely established in T2D-CKD subjects.

Keywords Homocysteine · Cysteine · Folate · Vitamin B12 · Type 2 diabetes · Chronic kidney disease

Introduction

Homocysteine is a metabolic intermediate in methyl group metabolism that is dependent on a number of nutritional B-vitamin cofactors. An emerging aspect of homocysteine metabolism is its relation to health and disease [1]. Perturbations of homocysteine metabolism, particularly intracellular, and subsequently circulating accumulation of homocysteine, i.e. hyperhomocysteinemia (hHcy), are associated with vascular disease risk as well as other pathologies, and the progression of chronic kidney disease (CKD) [1, 2]. Moreover, in type 2 diabetes (T2D), hHcy is strongly associated with increased risk of cardiovascular disease (CVD) and mortality [3]. Folic acid and vitamin B_{12} are vital in humans for several metabolic reactions, including the remethylation pathway. However, clinical studies have shown that folic acid therapy is not very effective in normalizing hHcy in uremic patients [4]. Indeed, it has been demonstrated that vitamin B_{12} supplementation alone, or in combination with folic acid, decreases total homocysteine (tHcy) concentrations, but

A. Pastore (✉) · G. Di Giovamberardino
Metabolomic and Proteomic Unit, Children's Hospital "Bambino Gesù", IRCCS, P.zza S. Onofrio, 4, 00165 Rome, Italy
e-mail: anna.pastore@opbg.net

A. Noce · N. Di Daniele
Department of Internal Medicine, Nephrology and Hypertension Unit, "Tor Vergata" University Hospital, Rome, Italy

A. De Stefano · R. Zenobi · M. Dessì
Department of Laboratory Medicine, "Tor Vergata" University Hospital, Rome, Italy

C. Callà
Department of Laboratory Medicine, "Policlinico Gemelli" University Hospital, Rome, Italy

Published online: 06 August 2014

full normalization is not achieved [5]. The authors reported also that supplementation with vitamin B_{12} decreases not only tHcy but also serum folate in patients with end-stage renal disease (ESRD) [5]. In a double-blind randomized controlled trial on patients with advanced CKD or ESRD, Jamison and collaborators showed that treatment with high doses of B-vitamins did not improve survival or reduce the incidence of vascular disease [6]. In our previous study, we demonstrated the importance of folate therapy and the secondary contribution of vitamin B_{12} in lowering tHcy in hemodialysis (HD) patients, and that this decrease correlated to the *MTHFR* genotype [7]. Furthermore, we reported that supplementation with both vitamin B_{12} and folate is useful only in HD patients with the wild-type genotype [7]. Most recently, Rafeq et al. [8] reported that even if hHcy is associated with diastolic dysfunction in patients with CKD, B-vitamin therapy did not improve heart failure (HF) outcomes despite lowering of plasma Hcy levels. Moreover, they showed that the therapy with vitamin B_{12} is associated with an increase in left atrial size, which is a surrogate for worsening left ventricular diastolic dysfunction, suggesting that high-dose B-vitamin therapy may be harmful in patients with CKD [8].

As data in the literature about B-vitamin Hcy-lowering therapy are conflicting, and since scant data on the correlation between endogenous levels of plasma tHcy, cysteine (Cys), folate and vitamin B_{12} in T2D patients with CKD are reported, we analysed tHcy and Cys levels in 178 T2D subjects at various stages of CKD in order to assess if there is a correlation between tHcy, Cys and endogenous folic acid and vitamin B_{12} levels and to verify the need for Hcy-lowering therapy in these patients.

Subjects and methods

Patient selection

All 178 patients were recruited from the Department of Internal Medicine, Nephrology and Hypertension Unit, Tor Vergata University Hospital, Rome (Italy). None of the patients was receiving folic acid and/or vitamin B_{12}. The control group ($n = 80$) was chosen from individuals who attended the outpatient clinic for minor problems (e.g. functional abdominal pain). Exclusion criteria were overweight, hypertension, dyslipidemia, hyperhomocysteinemia, and hyperglycaemia. CKD patients were divided into four groups, from I to IV, according to recently published guidelines [9]. Group 0 corresponds to T2D patients without renal disease. The estimated glomerular filtration rate (eGFR) was evaluated using the Chronic Kidney Disease Epidemiology Collaboration (CKD-EPI) formula [10].

Ethics

The study was carried out in accordance with the principles of the Declaration of Helsinki, and was approved by the Medical Ethical Committee of the Tor Vergata University Hospital. All participants in the study gave written informed consent.

Blood sampling and biochemical measurements

For both tHcy and Cys determinations, blood samples were collected into K_3EDTA Vacutainer Tubes (Becton–Dickinson, Franklin Lakes, NJ, USA), placed on ice, and immediately centrifuged and stored at −20 °C until analysis. For serum folate and vitamin B_{12} assays, blood samples were collected into Vacutainer Standard Separator Tubes (SST TM II Advance; Becton–Dickinson, Franklin Lakes, NJ, USA) in the dark, and immediately stored at −20 °C.

Vitamin B_{12} and folate concentrations were measured by an automated chemiluminescence system (Centaur®, Bayer AG, Leverkusen, Germany), following the manufacturer's instructions. tHcy and Cys levels were determined using the derivatization and chromatography procedures performed as previously reported [11]. All routine laboratory parameters were determined using Dimension Vista 1500 (Siemens, Healthcare Diagnostics, Milan, Italy).

Statistical analysis

All statistical analyses were performed with the statistical package SPSS for Windows (Ver. 8.0.0; SPSS). Kolmogorov–Smirnov and Lilliefors tests for normality were performed for all data. The 2-tailed nonparametric Mann–Whitney U test was used for comparison between CKD stage groups, and correlations were calculated as Spearman correlation coefficients. A value of $P < 0.05$ was considered statistically significant; $P < 0.01$ was considered extremely statistically significant.

Results

Table 1 summarizes the clinical and epidemiological features of 80 healthy subjects (control group, 35 males and 45 females), and of 178 diabetic patients divided into five renal failure stage subgroups, according to the recently published guidelines [9]. Total homocysteine levels evaluated in all T2D patients (33.42 ± 8.7 μmol/l) were significantly higher compared to healthy controls (8.3 ± 2.5 μmol/l, $P < 0.001$). tHcy concentrations were increased in all subgroups of T2D-CKD patients compared to controls; this

Table 1 Clinical and epidemiological features of the study population

	Controls (n = 80)	CKD stage					
		0 (n = 35)	I (n = 35)	II (n = 37)	IIIa (n = 16)	IIIb (n = 22)	IV (n = 33)
Sex (M/F)	35/45	26/9	17/18	17/20	9/2	21/6	17/16
Age (years)	74.0 ± 2.0	61.8 ± 1.1	68.6 ± 1.4	73.5 ± 0.8	72.0 ± 1.7	74.0 ± 1.5	74.9 ± 1.3
BMI (Kg/m^2)	19.0 ± 0.4	32.4 ± 0.8	30.3 ± 0.5	30.1 ± 0.6	29.0 ± 3.5	30.1 ± 5.2	29.6 ± 0.5
eGFR (ml/min/1.73 m^2)	117.0 ± 2.0	107.4 ± 3.0	99.8 ± 1.3	74.9 ± 0.9	53.5 ± 0.8	34.7 ± 0.6	21.9 ± 0.4
Albuminuria (mg/g)	9.3 ± 2.2	9.8 ± 2.8	87.7 ± 20.6	108.4 ± 35.3	149.0 ± 45.2	338.0 ± 102.5	628.0 ± 284.0
Creatinine (mg/dl)	0.72 ± 0.30	0.74 ± 0.10	0.80 ± 0.10	0.89 ± 0.14	1.30 ± 0.13	1.70 ± 0.25	2.55 ± 0.49
Glucose (mg/dl)	93 ± 6	144 ± 47	140 ± 44	123 ± 29	147 ± 39	146 ± 64	139 ± 62
Triglyceride (mg/dl)	102 ± 33	114 ± 47	130 ± 56	128 ± 52	163 ± 60	168 ± 62	219 ± 82
Total cholesterol (mg/dl)	160 ± 23	189 ± 26	178 ± 64	176 ± 32	171 ± 51	171 ± 34	177 ± 41
HbA_{1c} (%)	5.1 ± 0.6	7.5 ± 2.1	7.8 ± 1.0	7.0 ± 0.8	7.1 ± 1.0	7.4 ± 1.6	7.8 ± 1.5
Albumin (g/dl)	4.2 ± 0.3	3.8 ± 0.6	3.9 ± 0.7	4.1 ± 0.8	3.7 ± 0.6	3.9 ± 0.7	4.0 ± 0.8

Data are expressed as mean ± standard deviation (SD)

CKD chronic kidney disease, *BMI* body mass index, *eGFR* estimated glomerular filtration rate, *HbA_{1c}* glycated hemoglobin

Table 2 Hcy and Cys concentrations and B-vitamin levels in T2D-CKD patients at various stages of chronic kidney disease

	Controls (n = 80)	CKD Stage					
		0 (n = 35)	I (n = 35)	II (n = 37)	IIIa (n = 16)	IIIb (n = 22)	IV (n = 33)
tHcy (μmol/l)	8.3 ± 2.5	25.2 ± 8.9	25.6 ± 12.5	27.1 ± 13.2	38.7 ± 15.2	39.7 ± 16.0	45.9 ± 21.1
Cys (μmol/l)	394.0 ± 57.0	598.6 ± 125.5	596.9 ± 122.9	616.6 ± 140.0	669.0 ± 148.0	702.1 ± 120.3	838.2 ± 176.9
Folate (ng/ml)	9.5 ± 3.6	11.3 ± 4.4	10.0 ± 4.3	10.0 ± 4.1	9.3 ± 3.4	8.8 ± 3.6	8.5 ± 2.1
Vit. B_{12} (pg/ml)	555.2 ± 154.2	319.7 ± 153.5	315.2 ± 164.2	338.3 ± 170.6	307.0 ± 96.2	397.0 ± 132.3	471.0 ± 146.3

Data are expressed as mean ± standard deviation (SD)

Hcy homocysteine, *Cys* cysteine, *T2D* type 2 diabetes, *CKD* chronic kidney disease, *tHcy* total homocysteine

rise appears to be related to the CKD stage, reaching an 82 % increase in CKD stage IV (Tables 2, 3). Interestingly, tHcy was significantly increased also in T2D patients not affected by renal failure (stage 0) compared to controls (25.2 ± 8.9 vs. 8.3 ± 2.5 μmol/l, $P < 0.001$) (Tables 2, 3). Also Cys levels resulted higher in T2D-CKD patients than in healthy subjects (703.4 ± 191.1 μmol/l vs. 394.0 ± 57.0 μmol/l, $P < 0.001$). Cysteine seemed to follow an increasing trend in relation to the CKD stage, and this rise would appear to contribute not only to diabetes but also to the stage of renal failure (Tables 2, 3). No significant differences in folate levels were found in T2D patients (9.6 ± 3.9 ng/ml) when compared to controls (9.5 ± 3.6 ng/ml, $P = 0.870$). Our results showed an increase of folate concentrations at stage 0 with respect to controls (11.3 ± 4.4 vs. 9.5 ± 3.6 ng/ml, $P < 0.05$), but a decrease at stages IIIb and IV compared to stage 0 CKD ($P = 0.03$ and $P = 0.001$, respectively). These findings could be explained by the high inter-individual variability found for folate levels (Tables 2, 3).

Vitamin B_{12} levels of T2D patients (364.6 ± 149.2 pg/ml) were significantly lower compared to controls (555.2 ± 154.2 pg/ml, $P < 0.001$), but this finding is not an expression of vitamin B_{12} deficiency, in which plasma levels are below the 200 pg/ml. Moreover, this decrease does not appear related to the CKD stage. Indeed, at stage IV CKD, vitamin B_{12} levels were significantly higher than at all other stages ($P < 0.001$), with the only exclusion of stage IIIb CKD, in which the rise was not significant ($P = 0.061$). In any case, also at stage IV CKD, vitamin B_{12} levels were still significantly lower compared to controls (471.0 ± 146.3 vs. 555.2 ± 154.2 pg/ml, $P = 0.009$). Finally, we found a significant correlation between plasma tHcy and Cys levels (Fig. 1a), whereas no correlation was found between tHcy levels and either folate (Fig. 1b) or vitamin B_{12} (Fig. 1c) concentrations.

Discussion

Patients with CKD represent a unique cohort for studying the potential relationship between hHcy and HF given the increased prevalence of both of these disease states in this patient population [12, 13]. Elevated tHcy levels in patients

Table 3 Statistical correlation of Hcy, Cys and B-vitamin values between control group and various stage of CKD

	0	I	II	IIIa	IIIb	IV
CKD stage (tHcy)						
Control group (tHcy)	$P < 0.001$	$P < 0.001$	$P < 0.001$	$P < 0.001$	$P < 0.001$	$P < 0.001$
Stage 0		$P = 0.878$	$P = 0.479$	$P < 0.001$	$P < 0.001$	$P < 0.001$
Stage I			$P = 0.623$	$P = 0.004$	$P < 0.001$	$P < 0.001$
Stage II				$P = 0.007$	$P = 0.002$	$P < 0.001$
Stage IIIa					$P = 0.847$	$P = 0.229$
Stage IIIb						$P = 0.247$
CKD stage (Cys)						
Control group (Cys)	$P < 0.001$	$P < 0.001$	$P < 0.001$	$P < 0.001$	$P < 0.001$	$P < 0.001$
Stage 0		$P = 0.946$	$P = 0.568$	$P = 0.086$	$P = 0.003$	$P < 0.001$
Stage I			$P = 0.523$	$P = 0.075$	$P = 0.003$	$P < 0.001$
Stage II				$P = 0.228$	$P = 0.021$	$P < 0.001$
Stage IIIa					$P = 0.453$	$P = 0.002$
Stage IIIb						$P = 0.003$
CKD stage (folate)						
Control group (folate)	$P = 0.023$	$P = 0.520$	$P = 0.505$	$P = 0.838$	$P = 0.421$	$P = 0.139$
Stage 0		$P = 0.216$	$P = 0.199$	$P = 0.114$	$P = 0.030$	$P = 0.001$
Stage I			$P = 1.000$	$P = 0.569$	$P = 0.281$	$P = 0.075$
Stage II				$P = 0.552$	$P = 0.261$	$P = 0.063$
Stage IIIa					$P = 0.668$	$P = 0.315$
Stage IIIb						$P = 0.698$
CKD stage (vitamin B_{12})						
Control group (B_{12})	$P < 0.001$	$P < 0.001$	$P < 0.001$	$P < 0.001$	$P < 0.001$	$P = 0.009$
Stage 0		$P = 0.895$	$P = 0.640$	$P = 0.756$	$P = 0.057$	$P < 0.001$
Stage I			$P = 0.561$	$P = 0.857$	$P = 0.053$	$P < 0.001$
Stage II				$P = 0.501$	$P = 0.170$	$P < 0.001$
Stage IIIa					$P = 0.026$	$P < 0.001$
Stage IIIb						$P = 0.061$

Abbreviations, see previous table

$P < 0.05$ was considered statistically significant; $P < 0.01$ was considered extremely statistically significant

with CKD have been attributed to reduced renal clearance of circulating Hcy and impaired Hcy metabolism [14, 15]. Likewise, similar mechanisms of deteriorating renal function in HF have been proposed to be the link between HF and hHcy [16, 17]. Recently, Rafeq et al. [8] reported that even if hHcy is associated with diastolic dysfunction in patients with CKD, B-vitamin therapy did not improve HF outcomes despite its lowering of plasma Hcy levels. Moreover, they showed that therapy with vitamin B_{12} is associated with an increase in left atrial size, which is a surrogate for worsening left ventricular diastolic dysfunction, suggesting that high-dose B-vitamin therapy may be harmful in patients with CKD.

Our study shows an increase of plasma tHcy in T2D-CKD patients. Our data support the recent meta-analysis and Mendelian randomization analysis performed among 4,011 cases and 4,303 controls, that revealed strong evidence for a causal association of Hcy level with the development of T2D [18]. Moreover, it has been reported that the status of plasma homocysteine is associated with both the risk and severity of nephropathy in T2D [19]. Despite the fact that earlier studies had shown a decrease of tHcy levels after B-vitamin treatment, a lack of correlation between plasma Hcy and Cys, and endogenous levels of folate and vitamin B_{12} was found in our CKD-T2D patients. Furthermore, we found increased levels also of plasma Cys. As Cys is the rate-limiting precursor of glutathione (GSH), the major cellular antioxidant, this finding seems to be in contrast with the low GSH levels reported in several studies on T2D patients [20]. Transport of Cys across the cell membrane is essential for GSH synthesis. It has been demonstrated that Hcy could block the Cys transport at the level of the ASC transporter [21]. Sekhar and co-workers [22] showed indeed that Cys levels are diminished in T2D erythrocytes, most probably because of the block of the transporter. In addition, we found high levels of tHcy also in T2D patients at stage 0 of CKD. Our data seem to contrast the data reported in the literature, in which it was demonstrated that tHcy is lower or normal in T2D patients, whereas increased tHcy levels were found only when nephropathy is present [13]. We hypothesized that the observed increase found in our non-nephropathic

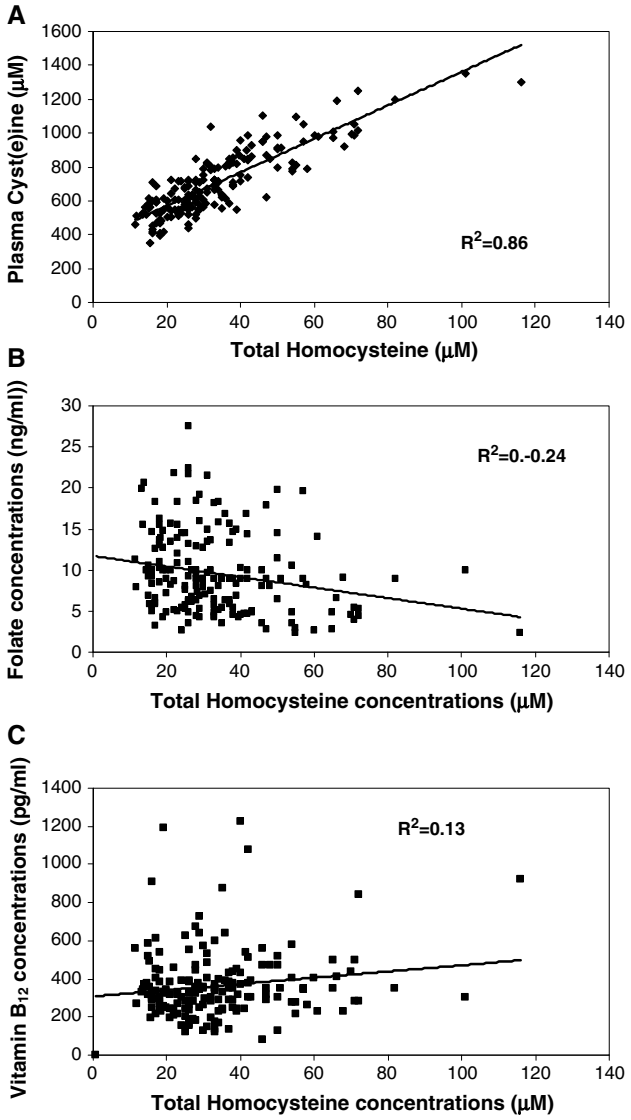


Fig. 1 Correlation between levels of various biomarkers found in T2D-CKD patients. Correlation between: plasma levels of tHcy and Cys (**a**), tHcy and folate (**b**), and tHcy and vitamin B_{12} (**c**) in T2D-CKD patients. Correlation coefficients (R^2) are reported. *T2D* type 2 diabetes, *CKD* chronic kidney disease, *tHcy* total homocysteine, *Cys* cysteine

T2D patients could be due to a masked defective function of kidney. In this case, tHcy may be considered an early biomarker of renal damage, even before the onset of microalbuminuria.

Folic acid and vitamin B_{12} are vital in humans for several metabolic reactions, including the remethylation pathway. However, clinical studies have shown that folic acid therapy is not very effective in normalizing hyperhomocysteinemia in uremic patients [4]. In a meta-analysis of randomized controlled trials, Pan and collaborators [22] reported that the estimated relative risk of CVD was not significantly different for cardiovascular events regardless of dialysis or in combination with B-vitamin therapy or the degree of reduction of Hcy levels, supporting the conclusion that Hcy-lowering therapy was not associated with a significant decrease in the risk for CVD events, stroke and all-cause mortality among patients with CKD.

Recently, both hHcy and vitamin B_{12} deficiency were found associated with diabetic retinopathy [23]. The authors reported that in their diabetic patients with retinopathy, vitamin B_{12} levels were significantly lower than in controls. Similarly to their results, in the present study we found decreased vitamin B_{12} levels in our T2D-CKD patients regardless of the CKD stage, although the concentrations found in our population were not an expression of vitamin B_{12} deficiency, in which plasma levels are below 200 pg/ml. The finding that at stage IV the vitamin B_{12} levels are significantly higher than in other stages of CKD is probably due to the reduced renal function in those patients, as it was demonstrated that excessive vitamin B_{12} is normally excreted in the urine [24]. We also found that folate levels were comparable to those of controls in our T2D-CKD patients. Taken together, these results seem to indicate the need for vitamin B_{12}, but not folate, therapy in type 2 diabetes patients with CKD.

Now that several studies have demonstrated that there are no benefits in terms of prevention of CVD events and mortality in individuals from treatment with B-vitamins, another concern that should be raised is about the safety of folic acid, particularly in relation to CVD and mortality risk. Small trials have been designed to deal with this negative effect. In a double-blind, placebo-controlled trial based on a total of 6,837 patients with ischemic heart disease (IHD), after a median 39 months of follow-up, it was observed that treatment with folic acid and vitamin B_{12} was associated with increased all-cause mortality [25]. In addition, in a multicenter, randomized, double-blind, placebo-controlled trial, House and collaborators found that high doses of B vitamins resulted in a greater decrease in glomerular filtration rate and an increase in vascular events such as myocardial infarction and stroke [26].

Considering this potential side effect, and as we did not find any correlation between tHcy levels and both endogenous folate and vitamin B_{12} concentrations, we believe that more large-scale clinical trials evaluating the effect of Hcy-lowering therapy on cardiovascular risk are needed to clarify whether Hcy-lowering therapy has the potential to clearly decrease cardiovascular risk in T2D-CKD patients.

Acknowledgments We are grateful to Dr. Olga Durante of the Nephrology and Hypertension Unit from the Tor Vergata University Hospital for helping us with the patient sample collection.

Conflict of interest The authors declare no conflict of interest. The authors declare that the results presented in this paper have not been published previously in whole or part, except in abstract format.

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