

**Review**

## Folic Acid and Homocysteine in Chronic Kidney Disease and Cardiovascular Disease Progression: Which Comes First?

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**Keywords**

Folate pathway · Homocysteine · Hyperhomocysteinemia · Cardiovascular disease · Chronic kidney disease

**Abstract**

**Background:** Hyperhomocysteinemia (Hhcy) occurs in about 85% of chronic kidney disease (CKD) patients because of impaired renal metabolism and reduced renal excretion. Folic acid (FA), the synthetic form of vitamin B<sub>9</sub>, is critical in the conversion of homocysteine (Hcy) to methionine. If there is not enough intake of FA, there is not enough conversion, and Hcy levels are raised. **Summary:** Hhcy is regarded as an independent predictor of cardiovascular morbidity and mortality in end-stage renal disease. Hhcy exerts its pathogenic action on the main processes involved in the progression of vascular damage. Research has shown Hhcy suggests enhanced risks for inflammation and endothelial injury which lead to cardiovascular disease (CVD), stroke, and CKD. FA has also been shown to improve endothelial function without lowering Hcy, suggesting an alternative explanation for the effect of FA on endothelial function. Recently, the role of FA and Hhcy in CVD and in CKD progression was renewed in some randomized trials. **Key Messages:** In the general population and in CKD patients, it remains a topic of discussion whether any beneficial effects of FA therapy are to be referred to its direct effect or to a reduction of Hhcy. While waiting for the results of confirmatory trials, it is reasonable to consider FA with or without methylcobalamin supplementation as appropriate adjunctive therapy in patients with CKD.

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## Introduction

Chronic kidney disease (CKD) represents an increasing burden on the worldwide healthcare system as it leads to poor outcomes and high costs. Reduced kidney function is associated with an increased risk for cardiovascular events and mortality compared with the general population [1], with incrementally increased risk as glomerular filtration rate declines [2].

Cardiovascular disease (CVD) is the most common cause of death in the setting of end-stage renal disease (ESRD) [3]. Of note, individuals with stage 3 CKD are more likely to die of CVD than to progress to ESRD. Furthermore, patients with combined cardiovascular and kidney disease are at much higher risk of mortality than patients with either in isolation [4].

As further confirmation of the existence of a narrow cross talk that may accelerate both processes, some CVD risk factors play, at the same time, also a role in the CKD progression and vice versa.

This association between CVD and CKD is present from the earliest stages of CKD; therefore, delay of ESRD remains a primary goal of CKD therapy simply because specific treatments to avoid CVD in this population currently do not exist. The mainstay of pharmacological treatment for CKD, aiming to slow progression to ESRD, is ACE inhibitors and angiotensin II receptor blockers for their hemodynamic/antihypertensive and anti-inflammatory/antifibrotic action [5].

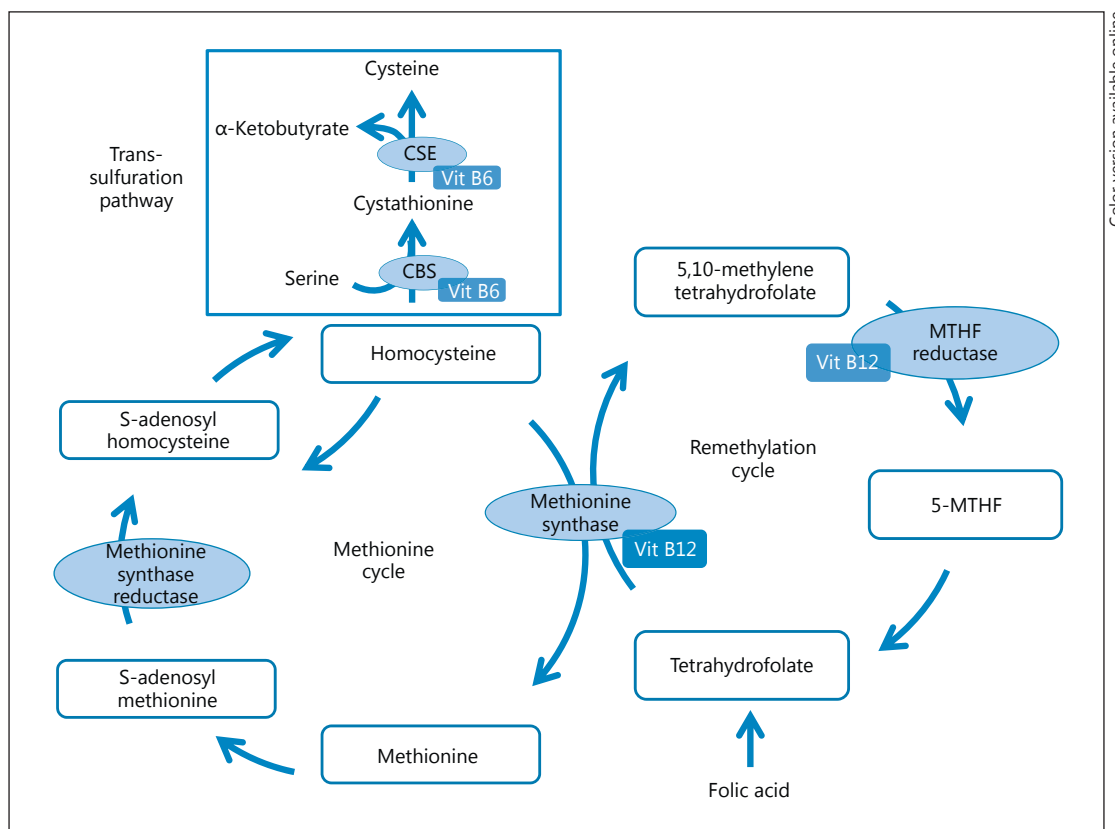
The increasing use of treatments to attenuate progressive CKD, most notably glycemic control in diabetic CKD and blood pressure treatment with ACE inhibitors and ARBs in almost all forms of CKD, have coincided with a plateau in the incidence of ESRD in the United States over the past few years. However, a constant incidence rate at over 100,000 per year cannot be a source of satisfaction (USRDS Annual Data Report 2014). The complex interactions among the different pathways of CKD progression also involving FGF-23 and Klotho, blunt the specific renoprotective effects of traditional RAAS-blocking agents [6]. However, new drugs and therapeutic approaches would be highly desirable to effectively slow the progressive renal function loss and hopefully reducing the cardiovascular burden of CKD patients.

The observation that individuals with very high blood levels of total homocysteine (tHcy; >100 mmol/L) caused by congenital errors of metabolism affecting cystathionine-B-synthase experience rapidly progressing atherosclerosis and thromboembolic events has led to the “homocysteine hypothesis,” which suggests that even a moderate increase in tHcy concentration may cause CVD [7].

Although the average tHcy level in the general population is about 10–15 mmol/L, depending on age, sex, and geographic region in patients with ESRD, it is on average 25–35 mmol/L [8, 9]. In various meta-analyses carried out in the general population tHcy level has been identified as a risk factor for CVD and mortality [10, 11]. Although in healthy subjects tHcy levels may be significantly reduced by supplementation with folic acid (FA), vitamin B<sub>12</sub>, and vitamin B<sub>6</sub>, results of randomized clinical trials (RCTs) of patients with CVD have been largely disappointing because neither the occurrence of cardiovascular events nor mortality was found to be reduced by treatment with vitamins [12–14]. However, the possible involvement of tHcy as a cardiovascular and mortality risk factor in patients with ESRD is a matter of debate since various study designs (retrospective, prospective observational, and vitamin intervention studies, meta-analyses) have resulted in conflicting results [15–17].

Furthermore, the high prevalence of hyperhomocysteinemia (Hhcy) in patients with CKD has generated interest in a potential role for Hhcy as a risk factor for progression of CKD [18, 19]. However, even for this outcome the results of previous trials have reported a null or harmful effect of supplementation with FA and B vitamins including cyanocobalamin [20, 21].

These conflicting results, after an initial nihilism, have led to a critical reappraisal of the effect of the changes of the metabolism of FA and homocysteinemia on CVD and CKD



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Fig. 1. Homocysteine metabolism.

progression that are deemed ultimately the result of a complex interplay between the effects of Hcy, FA, enzyme activity/gene variants, and FA fortification programs that exist in some countries.

### Homocysteine and FA Pathway

Homocysteine (Hcy), an amino acid not involved in protein synthesis, is an intermediate in methionine metabolism. Plasma Hcy levels are determined by several factors, such as genetic alterations of enzymes of methionine metabolism and the deficiency of vitamin B<sub>12</sub>, vitamin B<sub>6</sub>, and FA. FA is not biologically active and it requires the activity of the methylenetetrahydrofolate reductase (MTHFR) enzyme. FA is a substrate for cellular production of tetrahydrofolate, a precursor of 5-methyltetrahydrofolate (5-MTHF). MTHFR is a key regulatory enzyme involved in folate-dependent Hcy remethylation. It catalyzes the reduction of 5,10-methylenetetrahydrofolate to 5-MTHF that is necessary for normal methionine synthase (MTS) enzyme activity, in addition to being the natural circulating form of folate. Folate transfers 1-carbon moieties to various organic compounds by increasing S-adenosylmethionine levels [22]. 80–90% of circulating Hcy is protein bound, 10–20% of the tHcy is present as Hcy-cysteine mixed disulfide and Hcy (dimer of Hcy), and <1% is present in the free reduced form [23].

There are two main strategies that can be used to lower Hcy: oral administration of FA or 5-MTHF. In addition to folate, both vitamin B<sub>6</sub> and vitamin B<sub>12</sub> are necessary cofactors in Hcy metabolism [17]. Hcy is located at a branch-point of metabolic pathways: it is irreversibly

degraded via the transsulfuration pathway to cysteine or it is remethylated back to methionine (Fig. 1).

#### *Transsulfuration*

Transsulfuration is facilitated by the action of two vitamin B<sub>6</sub>-dependent enzymes: cystathionine β-synthase (CBS) and cystathionine γ-lyase (CTH). CBS catalyzes the condensation of Hcy and serine to cystathionine, and CTH subsequently catalyzes the hydrolysis of cystathionine to cysteine and α-ketobutyrate. Human CBS is expressed in the liver, kidneys, muscle, brain, and ovary, and also during early embryogenesis in the neural and cardiac systems [23].

#### *Remethylation*

Hcy remethylation to methionine is catalyzed by the MTS enzyme and links the folate cycle with Hcy metabolism. MTS requires vitamin B<sub>12</sub> as a cofactor and reactivates the complex by reductive methylation, using S-adenosylmethionine as a methyl donor [19]. While the MTS enzyme is ubiquitously expressed, another Hcy remethylation system, betaine-Hcy methyltransferase is mainly expressed in the liver and kidneys [23]. The function of the MTHFR enzyme is of great importance in the regulation of available 5-MTHF for Hcy remethylation.

### **MTHFR Gene**

MTHFR is a key enzyme in folate and Hcy metabolism and catalyzes the conversion of 5,10-methylenetetrahydrofolate into 5-MTHF, the predominant circulating form of folate. It has been demonstrated that several polymorphisms in genes involved in the Hcy-methionine pathway result in Hhcy, suggesting that such genetic variants may play a role in several multifactorial disorders associated with Hhcy of high prevalence in the general population.

Although several *MTHFR* gene variants have been identified, the most characterized and understood are the single nucleotide polymorphisms at position 677 (*MTHFR* 677C>T), at position 1298 (*MTHFR* 1298A>C), at position 1317 (*MTHFR* 1317T>C), and at position 1793 (*MTHFR* 1793G>A) [23, 24].

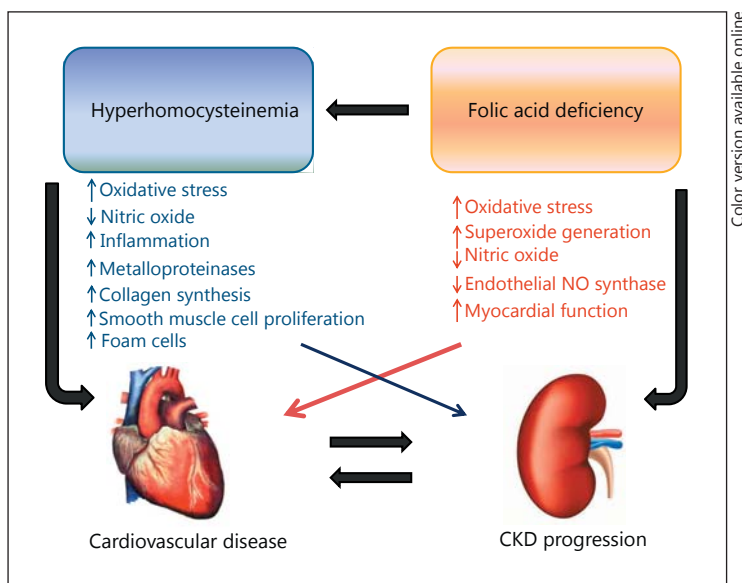
Bagley and Selhub showed that in red blood cells of *MTHFR* 677CC individuals, folate is totally represented by 5-MTHF. Conversely, the carriers of the 677TT genotype accumulate formylated folates, which points to a disruption of the folate cycle [25, 26]. The 677C>T substitution is the most common missense variation of *MTHFR*, with a global prevalence of 40% [25]. The frequency of this variant differs across the races: the TT homozygote genotype is approximately 1.45% in African blacks, 9.6% in Turkish, 12% in Indians, 41% in Chinese, and 23% in Italians [27, 28].

Although the main function of the MTHFR enzyme is to regulate the availability of 5-MTHF for Hcy remethylation, the pathological consequences of variants of *MTHFR* gene cannot be attributed solely to the increase in Hcy levels. Therefore, it is a matter of debate whether the cause of this association is the increase in Hcy levels or the FA deficiency or both.

### **Hhcy and CVD**

Hhcy occurs in about 85% of CKD patients because of impaired renal metabolism and reduced renal excretion [29, 30] and is regarded as an independent predictor of CVD morbidity and mortality in ESRD.

Hhcy exerts its pathogenic action on the main processes involved in the progression of vascular damage already enhanced in CKD patients. Hhcy induces oxidative stress and antag-



**Fig. 2.** The role of hyperhomocysteinemia in the atherosclerotic process.

onizes the vasodilator properties of NO by the formation of S-nitrosohomocysteine, thus leading to endothelial dysfunction [31]. Following oxidative injury, endothelial cells produce various cytokines participating in inflammatory reactions. The link between Hcy and inflammatory factors seems to be the activated transcription factor, nuclear factor-kappa B [32]. Hhcy activates metalloproteinases and induces collagen synthesis, leading to the reduction of vascular elasticity [33]. In addition to abnormal endothelial function, in mice, Hhcy causes vascular hypertrophy and remodeling, impairs vascular properties and increases stiffness of arteries or arterioles [34]. Hcy was proven to promote the proliferation of smooth muscle cells leading to several interactions with platelets, clotting factors, and lipids [35], and indeed might contribute to the scavenger receptor-mediated uptake of oxidized-LDL by macrophages resulting in foam cell formation in atherosclerosis [36]. These pathways end to amplify the atherosclerotic process and the inflammatory state present in CKD [37–41] (Fig. 2).

### Role of FA and B Vitamins in CVD

Endothelial dysfunction or damage is an early process in atherogenesis and a marker of vascular disease. There is a good agreement to support the theory that improvement in endothelial function decreases the risk of CVD. FA has also been shown to improve endothelial function without lowering Hcy, suggesting an alternative explanation for the effect of FA on endothelial function. FA improves endothelial function in coronary artery disease via mechanisms largely independent of Hcy lowering [42].

Vascular cells, particularly endothelial cells may be especially vulnerable to Hhcy, since they do not express CBS, the first enzyme of the hepatic reverse transsulfuration pathway, or betaine-Hcy methyltransferase, which catalyzes the alternate remethylation pathway in the liver using betaine as a substrate [43, 44]. Therefore, endothelial cells can eliminate Hcy only by the FA and vitamin B<sub>12</sub>-dependent remethylation pathway regulated by MTHFR and MTS. For this reason, normal activity of both MTHFR and MTS is essential to prevent the increase in Hcy to a pathological level in vascular endothelial cells [45].

In experimental models, FA improves endothelial function by reducing intravascular oxidative stress, intracellular superoxide generation, and by increasing the half-life of NO [46]. Indeed FA modulates endothelial NO synthase (eNOS) activity, regulating the bioavailability of tetrahydrobiopterin (BH<sub>4</sub>), the cofactor required for the eNOS enzyme activity [47, 48]. FA supports the biosynthesis of BH<sub>4</sub> from dihydrobiopterin (BH<sub>2</sub>) by proton and electron donation [49]. In mice, FA supplementation resulted in a significant improvement in myocardial function after artificial coronary artery occlusion [45] (Fig. 2).

Folate therapy reduces, but does not normalize Hcy levels, frequently high in CKD patients. The mechanisms of this folate resistance are not fully known, the entry of folate into the cell is mediated by specific folate receptors, whose expression is also modulated by folate status, through an Hcy-dependent regulation mechanism involving heterogeneous nuclear ribonucleoprotein-E1 (hnRNP-E1). In peripheral mononuclear cells of hemodialysis (HD) patients, FR2 expression is decreased and is not responsive to variations in Hcy concentration, while the intracellular machinery (receptor mRNA and hnRNP-E1), possibly triggering its regulation, is preserved [50].

### Hhcy and CVD Risk in ESRD Patients

Heinz et al. [16] in a meta-analysis of retrospective (11 studies including 1,506 individuals), prospective observational studies (12 studies including 1,975 individuals), and intervention trials (5 studies including 1,642 dialysis patients) found that tHcy level is a risk factor for both CVD and mortality in patients with ESRD who neither receive additional FA supplements nor live in regions with mandatory FA fortification. This effect was stronger in prospective than retrospective studies. The prospective studies included in the meta-analysis showed that in unsupplemented patients with ESRD, an increase of 5 mmol/L in tHcy concentration is associated with an increase of 7% in the risk of total mortality and an increase of 9% in the risk of cardiovascular events.

In contrast, in a randomized prospective study performed on 341 HD patients, we failed to assess any role for baseline Hhcy and MTHFR polymorphism as independent risk factors for overall and cardiovascular mortality [17].

Similarly to the reverse epidemiology: higher mortality in HD patients with low cholesterol, 2 studies showed that patients with very low Hcy plasma levels had worse outcomes including a higher incidence of hospitalization and mortality [51, 52]. This raises the question as to whether in uremic patients Hhcy is consequential rather than causal in the cardiovascular complications [53]. Alternatively, Hcy could be an innocent bystander, or a surrogate of the real culprit. The latter possibility leads us to the search for potential alternative candidates. First, the accumulation of Hcy in blood leads to an intracellular increase in S-adenosylhomocysteine, a powerful competitive methyltransferase inhibitor, which by itself is considered a predictor of cardiovascular events. DNA methyltransferases are among the principal targets of Hhcy, as assessed in experimental and clinical studies. In CKD and in uremia, Hhcy and high intracellular S-adenosylhomocysteine are present and are associated with abnormal allelic expression of genes regulated through methylation, such as imprinted genes, and pseudoautosomal genes, thus pointing to epigenetic dysregulation. These changes are susceptible to reversal upon Hcy-lowering therapy obtained through folate administration. Furthermore, it has to be kept in mind that Hcy is mainly protein-bound, and its effects could therefore be linked to protein homocysteinylation. In this respect, increased protein homocysteinylation has been found in uremia, leading to impairments in protein function [54].

Despite their higher risk of CVD, CKD diabetic patients tend to have lower Hcy levels than nondiabetic patients whatever the degree of chronic renal insufficiency. This finding is considered to be secondary to an impairment of transmethylation pathway that occurs as a result of both impaired folate utilization and functional vitamin B<sub>12</sub> deficiency, paradoxically leading to a reduction in serum creatinine and Hcy levels [55]. Hyperglycemia itself may increase Hcy level poorly attenuated by FA therapy [37].

### Folate and Vitamin B: Interventional Studies on CVD in CKD Patients

Folate therapy reduces, but does not normalize Hcy levels; often increased in CKD, the higher the baseline Hcy level, the better its response to treatment. However, the results of interventional studies are conflicting.

Furthermore, the optimum dose of FA and vitamin B supplementation needed to prevent CVD in ESRD patients remains uncertain, ranging from 2.5 to 5 mg of FA three times a week up to more than 15 mg/day. The simultaneous administration of intravenous B complex vitamins is more efficient in reducing Hcy serum levels and restoring the remethylation pathway [37]. To our knowledge, there is a small number of interventional studies that have analyzed the effect of Hcy-lowering agents in early stages of CKD.

A combined treatment consisting of pravastatin, vitamin E, and Hcy-lowering agents reduced blood pressure, carotid intima-media thickness and urinary albumin excretion and increased brachial artery flow-mediated dilatation [56].

In the meta-analysis of intervention trials with B vitamins by Heinz et al. [16], there was a significant risk reduction for CVD (relative risk, 0.73;  $p = 0.02$ ), but no risk reduction for total mortality or the composite end point including total mortality (relative risk, 1.01;  $p = 0.9$ ).

A further meta-analysis included 3,886 patients with patients with ESRD or advanced CKD (creatinine clearance, <30 mL/min) from 7 qualified randomized trials aimed to assess the relationship between FA therapy (with or without vitamin B<sub>6</sub> and B<sub>12</sub>) with CVD reported as one of the end points. FA therapy was shown to reduce the CVD risk by 15% in ESRD patients; a greater benefit was seen in those treated for longer than 24 months and in those from areas with no or partial grain fortification. Reducing Hcy level by more than 20% after treatment was also beneficial in reducing CVD risk independently of FA fortification [57].

In a randomized prospective study performed on 341 HD patients, we investigated whether supplementation with 5-MTHF versus FA treatment could affect patient survival. We found an improvement in the survival rate of patients treated with 5-MTHF, although there was no difference in Hcy levels between the two groups of therapy [17].

These findings were not confirmed in other studies. In a large double-blind RCT with 2,056 participants with advanced CKD or ESRD and high Hcy levels followed up for 3.2 years, treatment with high doses of FA and B vitamins did not improve survival or reduce the incidence of vascular disease [21].

A meta-analysis of 10 studies concluded that Hcy-lowering therapy is not associated with a significant decrease in the risks for CVD events, stroke, and all-cause mortality among patients with CKD [58]. The deviation from the previous meta-analysis may reflect the enrollment in some RCTs of a high number of DM participants and their performance in grain fortification areas.

Several post hoc analyses have shown that numerous factors including age, baseline tHcy levels, FA fortification of grains, B<sub>12</sub> status, renal function, comorbidities, and medications may modify the effect of B vitamin therapy on vascular risk in individuals with high tHcy.

### Hhcy, FA, and Renal Disease

The renal damage could be related to the same pathways activated on the cells of the vascular wall by Hhcy and by reduced availability of FA and able to promote the cardiovascular damage. Furthermore, they amplify the chronic inflammation of CKD patients. Inflammation, in addition to being an important link between CKD and CVD, is involved in CKD progression [59].

Observational studies have shown that Hhcy is associated with the risk of developing CKD and albuminuria [60, 61].

In contrast, previous trials have pointed out a null or harmful effect of supplementation with FA and B vitamins including cyanocobalamin. In the HOST study, the treatment with high doses of FA (40 mg/day) and other B vitamins (vitamin B<sub>6</sub>, 100 mg/day; vitamin B<sub>12</sub>, 2 mg/day) did not improve survival or delay the time to initiating dialysis in patients with advanced CKD or ESRD [20]. The DIVINE trial showed that treatment with FA (2.5 mg/day) and B vitamins (vitamin B<sub>6</sub>, 25 mg/day; cyanocobalamin, 1 mg/day) resulted in a greater decrease in GFR and an increase in vascular events in 238 patients with diabetic nephropathy [20]. A recent study on 630 Italian Caucasian population found a lower frequency of *MTHFR* 677C>T and A1298A>C polymorphisms among dialysis patients in end-stage kidney failure compared to subjects without or with slight-moderate renal impairment, suggesting a protective role of both polymorphisms on renal function [62].

### Hcy, FA, and Kidney Transplantation

In kidney transplant recipients, time of dialysis, anemia, and chronic immunosuppression are likely to trigger a combination of immunologic responses, prothrombotic state, dysmetabolic alterations, and inflammatory abnormalities, which lead to a substantially increased cardiovascular risk in comparison with the general population [63].

Treatment of stable kidney transplant recipients with multivitamins containing high-dose FA, B<sub>6</sub>, and B<sub>12</sub> lowers tHcy levels but does not reduce CVD outcomes or total mortality in this patient population [64].

### Future Perspectives

Recently, the interest about the role of FA and Hhcy in CVD and in CKD progression was renewed following the publication of a meta-analysis and 2 large randomized trials.

In a large meta-analysis of genetic studies and clinical trials, it was stated that the efficacy of FA therapy in stroke prevention should be assessed taking into account combined effects of baseline folate levels, *MTHFR* gene C677T polymorphism and the involvement in the studies of countries where there is or not a FA fortification program [65].

The China Stroke Primary Prevention Trial (CSPPT), a large randomized trial among adults with hypertension without a history of stroke or MI, was designed, using individual measures of *MTHFR* genotype and baseline folate level, to test the hypothesis that there would be a larger effect of FA intervention in an Asian region without an FA fortification program. The CSPPT found that the ACE inhibitors plus FA therapy, compared with ACE inhibitors alone, significantly reduced the relative risk of first stroke by 21%. Among individuals with CC or CT genotypes, the highest risk of stroke and the greatest benefit of FA therapy were in those with the lowest baseline folate levels. In addition, individuals with the TT genotype may require a higher dosage of FA supplementation to overcome biologically insufficient levels [66].



A prespecified renal substudy of the CSPPT examined the effects of a combined treatment of ACE inhibitors and FA versus ACE inhibitors alone in reducing the risk of renal function decline in a hypertensive population [67]. The study was conducted in a population without FA fortification, including participants across a spectrum of renal function at baseline from normal to moderate CKD, and that cyanocobalamin was not used in the therapy. The authors found that treatment with ACE inhibitors plus FA, as compared with ACE inhibitors alone, reduced the risk of progression of CKD by 21% and the rate of eGFR decline by 10% in hypertensive patients. Patients with CKD benefited most from the FA therapy, with a 56 and 44% reduction in the risk for progression of CKD and the rate of eGFR decline, respectively. In the ACE inhibitors plus FA group, both the size of the increase in serum folate and the drop in Hcy were greater in the participants with CKD than in those without CKD. In particular, the greatest drop in serum Hcy was in TT homozygotes of MTHFR C677T polymorphism, while the magnitude of the declines in those with CC/CT genotypes was relatively small. Similarly to the stroke risk found in CSPPT, the effect of the MTHFR genotype on CKD progression is expected to be subject to modification by population dietary folate levels.

Moreover, the authors performed exploratory subgroup analysis to assess the treatment effect on the primary outcome in various subgroups among participants with CKD: the reduction in the risk of CKD was observed in the subgroup with diabetes.

The opposite results seen in these trials compared with previous studies may be partly explained by differences in patient characteristics and treatment schemes among these studies. Baseline FA levels may have an impact on the efficacy of the FA intervention therapy. Both trials were made in a population without FA grain fortification, while the other 2 studies were carried out in countries with FA fortification programs.

The efficacy of FA therapy found in the 2 trials may be influenced by the degree of cardiovascular and renal impairment. It is a matter of fact that the CSPPT included adults without a history of stroke and myocardial infarction. In relation to the renal substudy of CSPPT, it must be pointed out that the participants were those with mild-to-moderate CKD, while the HOST study enrolled patients with advanced CKD or ESRD.

Confirmation of the findings found in both trials requires studies in other countries without FA fortification. The impact of FA therapy on diabetic patients with or without CKD requires clarification.

Furthermore, in consideration of substantial variability in blood folate levels found also in countries with FA fortification and the widespread use of FA supplements [67], the results of the 2 trials suggest that it may be possible to further reduce stroke incidence and CKD progression using more targeted FA therapy, in particular, among those with the TT genotype and low or moderate folate levels. Apart from these considerations, both in the general population and in CKD patients, it remains a topic of discussion whether any beneficial effects of FA therapy are due to its direct effect or to a reduction of Hhcy.

While waiting for the results of confirmatory trials, it is reasonable to consider FA with or without methylcobalamin supplementation as appropriate adjunctive therapy in patients with CKD. For patients with early CKD who do not need to restrict their intake of potassium or phosphorus, this could come in the form of a healthy diet rich in natural sources of folate, and by use of FA supplementation, once the folate status/levels have been verified, in the more advanced stages of CKD.

### Disclosure Statement

There are no conflicts of interest to disclose.

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