Folic Acid, Vitamin B12 and Chronic Kidney Disease

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Patients affected by chronic kidney disease (CKD) or end-stage renal disease (ESRD) experience a huge cardiovascular risk and cardiovascular events represent the leading causes of death. Folic acid and vitamin B12 could not only be mere cofactors in the homocysteine metabolism; they may have a direct action in determining tissue damage and cardiovascular risk.

cardiovascular disease | chronic kidney disease | end-stage renal disease | hyperhomocysteinemia | folic acid | vitamin B12

1. Introduction

Patients affected by chronic kidney disease (CKD) or end-stage renal disease (ESRD) have a shorter life expectancy than those with normal renal function, primarily due to the dramatic increase in cardiovascular mortality [1]. Chronic hemodialysis treatment is associated with a 10 to 50-fold higher risk of premature death than in the general population, and cardiovascular disease (CVD) represents the leading cause of death in hemodialysis patients [2][3]. Nevertheless, such increased cardiovascular risk is present since earlier stages of CKD [4].

In randomized clinical trials (RCTs), the traditional Framingham factors, such as hypertension, dyslipidemia and diabetes mellitus have been proven to be poor predictors of cardiovascular risk in this population. Therefore, there has been growing attention on non-traditional cardiovascular risk factors, in particular oxidative stress, endothelial dysfunction, chronic inflammation, vascular calcification Chronic Kidney Disease—Mineral and Bone Disorder (CKD-MBD) and hyperhomocysteinemia [5].

The “homocysteine hypothesis” arises from the observation that subjects with very high homocysteine blood levels due to congenital homocysteine metabolism impairment are more susceptible to develop a severe form of progressing atherosclerosis. Thus, over the years, research has been conducted into the possible link between an even moderate rise in homocysteine levels and cardiovascular risk and mortality, with conflicting results [6][7].

Although patients with CKD and ESRD display elevated homocysteine levels, the role of hyperhomocysteinemia as a cardiovascular and mortality risk factor in this population is still to be fully elucidated and deserves further investigation [8][9][10][11][12].

Furthermore, the high prevalence of hyperhomocysteinemia in patients with CKD has increased interest in speculating the role for hyperhomocysteinemia as a risk factor for the progression of CKD [13][14].

The role of folic acid and vitamin B12 role is well recognized, as they are not only essential cofactors for homocysteine metabolism, but their homeostasis disruption may be related directly to cardiovascular risk and CKD progression [11][15].

2. B Vitamins—Homocysteine Pathway

B vitamins, including vitamin B9 (folate) and vitamin B12 (cobalamin) are water-soluble vitamins involved in several normal cellular functions: they are providers of carbon residues for purine and pyrimidine synthesis, nucleoprotein synthesis and maintenance in erythropoiesis [16].

Folic acid is derived from polyglutamates that are converted into monoglutamates in the bowel, and then transported across mucosal epithelia by a specific carrier. The circulating form of folic acid is 5-methyltetrahydrofolate (5-MTHF) [17].

Vitamin B12, ingested with nutrients such as cobalamin, complexes with salivary haptocorrin, and is released abruptly from cobalamin by pancreatic proteases in the duodenum. Then, cobalamin, binds to an intrinsic factor secreted from the parietal cells of the stomach: when this complex arrives at the distal ileum, it is endocytosed from the enterocytes through cubilin. Then, cobalamin is carried into the plasma by a plasma transport protein named transcobalamin [16]. B12 is filtered by the glomerulus; however urine excretion is minimal due to reabsorption in the proximal tubule.

In target tissues, cobalamin is metabolized into two active forms: adenosylcobalamin in the mitochondria and methylcobalamin in the cytosol. Methylcobalamin is a methyl-transferring cofactor to the enzyme methionine synthase allowing homocysteine remethylation to methionine [17].

Homocysteine is a thiol-containing amino acid, not involved in protein synthesis, deriving from methionine metabolism. Plasma levels of homocysteine depend on several factors, such as genetic alteration of methionine metabolism enzymes or deficiency of vitamin B12, vitamin B6 or folic acid [18].

Methionine is transformed into S-adenosylmethionine (SAM) and then converted in S-adenosylhomocysteine (SAH) through a reaction catalyzed by methionine synthase reductase (MTRR). SAM, one of the most important methyl group donors, is formed within mitochondria and is a cofactor for a mutase known as methylmalonyl-CoA-mutase. This enzyme converts methylmalonyl-CoA into succinyl CoA, representing a crucial step in the catabolism of various amino acids and fatty acids. These processes also require pyridoxine (vitamin B6) as a cofactor [18].

Homocysteine is the final product derived from hydrolysis of SAH to homocysteine and adenosine. Metabolism of homocysteine includes two different pathways: remethylation and transsulfuration (**Figure 1**A). In the remethylation

pathway, methionine is regenerated through a reaction catalyzed by the enzyme methionine synthase (MTS), requiring folate and vitamin B12 as cofactors. Given that folate is not biologically active, it necessitates transformation into tetrahydrofolate that is then converted into methylenetetrahydrofolate (MTHF) by the enzyme methylenetetrahydrofolate reductase (MTHFR) [19].

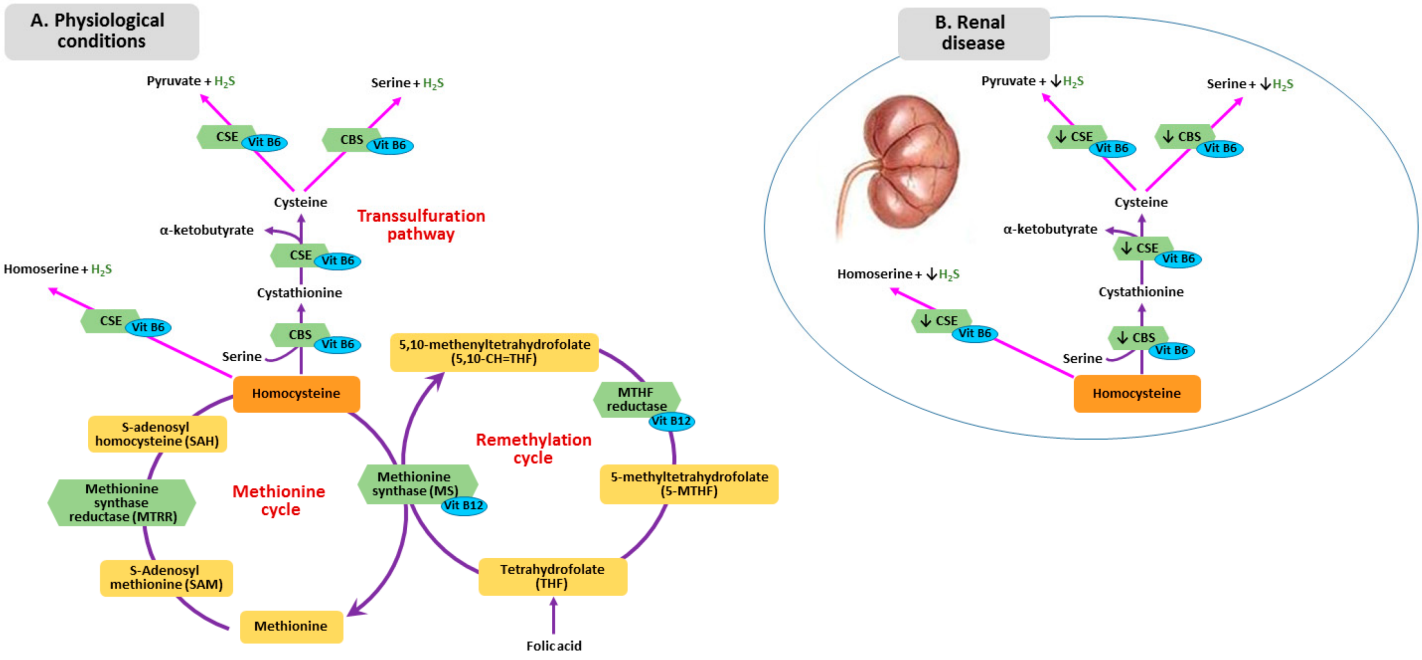

Figure 1. Homocysteine Metabolism in physiological condition (**A**) and in renal disease (**B**). CSE: cystathionine gamma-lyase; CBS: cystathionine beta synthase.

The other pathway responsible for the homocysteine metabolism is transsulfuration. First, homocysteine combines with serine forming cystathionine by cystathionine beta synthase (CBS), then, cystathionine is hydrolyzed into cysteine and α-ketobutyrate by cystathionine γ-lyase (CTH) Human CBS is expressed in the liver, kidneys, muscle brain and ovary, and during early embryogenesis in the neural and cardiac systems [20].

The sulfur atom, in the form of sulfane sulfur or hydrogen sulfide (H_2S), can be involved in vitamin B12-dependent methyl group transfer [21][22]. Alterations in methylation pathway, which causes a reduction of proteins and DNA methylation, results in abnormal vascular smooth muscle cell proliferation and increased lipid peroxidation [23]. Sulfur is a side product of conversion of homocysteine to cysteine by the enzymes CBS and cystathionine gamma-lyase (CSE). H_2S is an angiogenic agent with antioxidant and vasorelaxing properties. Moreover, H_2S represents an endogenous gaseous mediator, similarly to nitric oxide (NO) and carbon monoxide [24], which plays a role in several physiological processes, namely vascular smooth muscle relaxation, inhibition of vascular smooth muscle cell proliferation and blood pressure lowering [25]. Li et al. proved that H_2S metabolism impairment might contribute to the development of uremia-associated accelerated atherosclerosis in CKD patients with diabetic nephropathy [26]. Patients with CKD and ESRD show lower H_2S plasma levels, which can result from downregulation of CBS and CSE, mediated by hyperhomocysteinemia (**Figure 1**B). Whether this phenomenon can be attributed to additional factors is still unclear [21].

Homocysteine can be found in reduced and oxidized form in the bloodstream: more than 90% of the total plasma homocysteine is oxidized and bound to proteins, while the remaining oxidized homocysteine exists as a disulfide form. Only 2% of the total homocysteine in plasma is present as a free reduced form [27].

Normal homocysteine plasmatic level is <10 mmol/L, concentrations >10; however, levels <16 mmol/L are defined as mild hyperhomocysteinemia, while severe hyperhomocysteinemia is diagnosed when homocysteine >100 mmol/L [28].

Homocysteine is minimally eliminated by the kidney, since in physiological conditions, only non-protein bound homocysteine is subjected to glomerular filtration, and then for most part reabsorbed in the tubuli and oxidized to carbon dioxide and sulfate in the kidney cells [25].

Moreover, in the kidney, homocysteine is above all transsulfurated and deficiency of this renal transsulfuration contributes to the elevation of plasma homocysteine [18].

3. Metabolism of Homocysteine, Folic Acid and Vitamin B12 in CKD

Patients with CKD and ESRD have been shown to have higher homocysteine blood levels compared to the general population [8][29]. It has been hypothesized that hyperhomocysteinemia in these patients may be induced by the abnormality of homocysteine metabolism in the kidneys rather than by reduced glomerular filtration rate. In fact, although free homocysteine can pass the ultrafiltration barrier due to its low molecular weight, it circulates in the bloodstream mostly (about 90%) in the protein-bound form [27]. In particular, transsulfuration and remethylation pathways occurring in the kidney may be affected by renal disease. Stable isotope studies in nondiabetic and diabetic patients with CKD have shown impaired metabolic clearance of homocysteine determined by dysfunction in both pathways [30].

In both CKD and ESRD patients, several metabolic alterations, including acidosis, systemic inflammation and hormonal dysregulation, together with comorbidities and multidrug therapies, can lead to malnutrition with subsequent folic acid and vitamin B12 deficiency. In addition, anorexia, gastroparesis, slow intestinal transit or diarrhea, increased gut mucosal permeability and gut microbiota impairment may represent worsening factors [31] [32].

Folic acid metabolism is impaired in uremic patients. Organic and inorganic anions, whose clearance is reduced in CKD, inhibit the membrane transport of 5-MTHF, thus compromising the incorporation into nucleic acids and proteins. Data suggest that transport of folates is slower in uremia and this implicated that, even with normal plasmatic folate levels, the uptake rate of folates into tissues may be altered [33]. In fact, serum folate concentration does not represent a reliable measure of tissue folate stores, but rather reflects recent dietary intake of the vitamin. Erythrocyte folate concentration is a better indicator of whole folate status. In a population of 112 dialysis patients,

Bamonti et al. found serum folate levels normal in only 37% of cases, despite over 80% of red blood cells folate levels within the normal range [34].

Regarding vitamin B12, several studies have shown a correlation between low serum vitamin B12 concentrations and high BMI, insulin resistance, type 2 diabetes, dyslipidemia and CVD [35]. Vitamin B12 in the blood is primarily protein-bound. Approximately 20% of circulating B12 is bound to transcobalamin: this is the biologically active form that can be taken up into cells. Although CKD patients display increased transcobalamin levels, they show an impaired vitamin tissue uptake of B12 [36]. Moreover, in uremic patients a functional vitamin B12 deficiency can be observed because of increased transcobalamin losses in the urine and reduced absorption in the proximal tubule. This can lead to a “paradoxical” increase in cellular homocysteine levels despite normal total B12 [37].

On the other hand, potentially overdosage-related vitamin B12 toxicity could result exacerbated in individuals with CKD. Cyanocobalamin, the most commonly used form of B12 supplementation therapy, is indeed metabolized to active methylcobalamin, releasing small amounts of cyanide whose clearance is reduced in CKD [34]. Under normal conditions, methylcobalamin is required to remove cyanide from the circulation through conversion to cyanocobalamin. However, in CKD patients, the reduced cyanide clearance prevents conversion of cyanocobalamin to the active form and therefore supplementation is less effective [38].

The appropriate range of B12 levels in CKD remains to be defined adequately. Downstream metabolites, such as methylmalonic acid and homocysteine, may more accurately reflect functional B12 status in uremic patients [35].

4. Homocysteine-Mediated Tissue Damage

The pathogenic role of hyperhomocysteinemia on cardiovascular system in CKD and ESRD is related to atherosclerosis progression in the context of an already enhanced risk of vascular damage determined by uremic syndrome. One possible mechanism is the induction of local oxidative stress, generating Reactive Oxygen Species (ROS) because of the thiol group, which rapidly undergoes autoxidation in the presence of oxygen and metal ion. Besides, hyperhomocysteinemia promotes Nicotinamide Adenine Dinucleotide Phosphate (NADPH) oxidase activity with further increase in ROS generation. Hyperhomocysteinemia also determines Nitric Oxide (NO) metabolism impairment in endothelial cells (including Nitric Oxide Synthase expression, localization, activation, and activity) leading, together with ROS-induced local microinflammation, to endothelial dysfunction [39].

In cultured endothelial cells, hyperhomocysteinemia has been shown to upregulate monocyte chemotactic protein 1 (MCP-1) and interleukin-8 (IL-8) production, resulting in monocyte adhesion to the endothelium [40]. The link between homocysteine and inflammatory factors seems to be the activated transcription factor NF-κB (nuclear factor kappa-light-chain-enhancer of activated B cells) [41].

Additionally, hyperhomocysteinemia induces vascular smooth muscle cells (VSMC) proliferation by promoting the expression of adhesion molecules, chemokine and VSMC mitogen leading to several interactions with platelets, clotting factors and lipids [42], and might contribute to the scavenger receptor-mediated uptake of oxidized- Low

Density Lipoprotein (LDL) by macrophages, triggering foam cell formation in atherosclerotic plaque [43][44][45][46]. Hyperhomocysteinemia also determines a vascular remodeling process that involves activation of metalloproteinase and induction of collagen synthesis, with subsequent reduction of vascular elasticity [47].

Likewise, elevated blood levels of homocysteine can cause endothelial reticulum stress with increase endothelial apoptosis and inflammation through a process mediated by ROS production and NF-κB activation [48][49][50]. Endothelial cells are known to be particularly vulnerable to hyperhomocysteinemia, since they do not express CBS, the first enzyme of the hepatic reverse transsulfuration pathway, or betaine-homocysteine methyltransferase (BHMT), which catalyzes the alternate remethylation pathway in the liver using betaine as a substrate [51].

Lastly, N-homocysteinylation of proteins is one process responsible for homocysteine toxicity, since it causes structural and functional loss. For LDL, homocysteinylation produces aggregation, accumulation of cholesterol and formation of foam-cells. Fibronectin is also involved in N-homocysteinylation: this reaction contributes to extracellular matrix remodeling, promoting the development of sclerotic processes [52].

Some peculiar effects of hyperhomocysteinemia on renal tissue have been described. Homocysteine can act directly on glomerular cells inducing sclerosis, and it can initiate renal injury by reducing plasma and tissue level of adenosine. Decreased plasma adenosine leads to enhanced proliferation of VSMC, accelerating sclerotic process in arteries and glomeruli. In a rat model of hyperhomocysteinemia induced by a folate-free diet, glomerular sclerosis, mesangial expansion, podocyte dysfunction and fibrosis occurred due to enhanced local oxidative stress. After treatment of the animals with apocynin, a NADPH oxidase inhibitor, glomerular injury was significantly attenuated [53].

5. Folic Acid and Vitamin B12 Impairment and Tissue Injury

Both folic acid and vitamin B12 have shown a potential direct relationship with cardiovascular outcomes with mechanism unrelated to homocysteine levels, although not clearly understood [54].

Folic acid improves endothelial function without lowering homocysteine, suggesting an alternative explanation for its effect on endothelial function that is possibly related to its anti-inflammatory, anti-oxidative and anti-apoptotic properties [55][56][57]. Experimental models revealed that folic acid can reduce endothelial dysfunction through the limitation of oxidative stress generation and the increasing of NO half-life [17]. 5-MTHF, the circulating form of folic acid, acutely improves NO-mediated endothelial function and decreases superoxide production. Moreover, 5-MTHF prevents oxidation of BH4 increasing enzymatic coupling of eNOS, enhancing NO production. Because 5-MTHF is a reduced form of folic acid that does not require conversion by dihydrofolate reductase, some direct effects may be attributable to redox mechanisms that are not seen when oral folic acid is used to increase plasma folate levels [58][59].

Doshi et al. investigated the direct effects of folic acid on endothelial function in patients with coronary artery disease (CAD) through Flow Mediated Dilatation (FMD) measurement before and after folic acid intake. FMD

improved at 2 h in parallel with folic acid blood concentration, while homocysteine blood level did not change significantly. These data suggest that folic acid improves endothelial function in CAD acutely by a mechanism largely independent of homocysteine [60]. Other authors demonstrated that high-dose folic acid (5 mg/day) improves endothelial function in CAD patients with an action not related to homocysteine level [60][61][62][63]. We have previously reported that supplementation with 5-MTHF versus folic acid improved survival rate without differences in homocysteine levels [11]. Pan et al. recently showed that folic acid treatment can inhibit atherosclerosis progression through the reduction of VSMC dedifferentiation in high-fat-fed LDL receptor-deficient mice [64].

From the vitamin B12 side, patients with chronic inflammation, such as the hemodialysis population, display decreased production of transcobalamin II, due to impaired uptake of circulating B12 by peripheral tissues. This can determine increased synthesis of transcobalamins I and III that brings to further accumulation of B12 in blood [65][66][67][68]. Therefore, in the context of inflammatory syndromes, despite high vitamin B12 blood levels, there is a vitamin B12 deficiency in target tissues, potentially leading to hyperhomocysteinemia and increased cardiovascular risk [69].

Concerning anemia, unless CKD and ESRD patients show significant folate depletion, additional supplementation of folic acid does not appear to have a beneficial effect on erythropoiesis or on responsiveness to Recombinant Human Erythropoietin (rHuEPO) therapy. However, a diagnosis of folate deficiency should be considered in such patients when significant elevation in mean cell volume or hypersegmented polymorphonuclear leucocytes are found, especially in subjects with malnutrition, history of alcohol abuse, or in patients hyporesponsive to rHuEPO. Measurements of circulating serum folate do not necessarily mirror tissue folate stores, and red blood cell folate measures provide a more accurate picture. Low red blood cells folate concentrations in these patients suggest the need for folate supplementation [70].

In patients with CKD, folate and vitamin B12 deficiency may represent an influential factor in renal anemia and hyporesponsiveness to rHuEPO therapy. As such, the possibility and the requirement of a regular supplementation is still a matter of debate [71].

Figure 2 illustrates the pathways involved in the amplification of atherosclerosis and inflammation triggered by hyperhomocysteinemia in CKD patients.

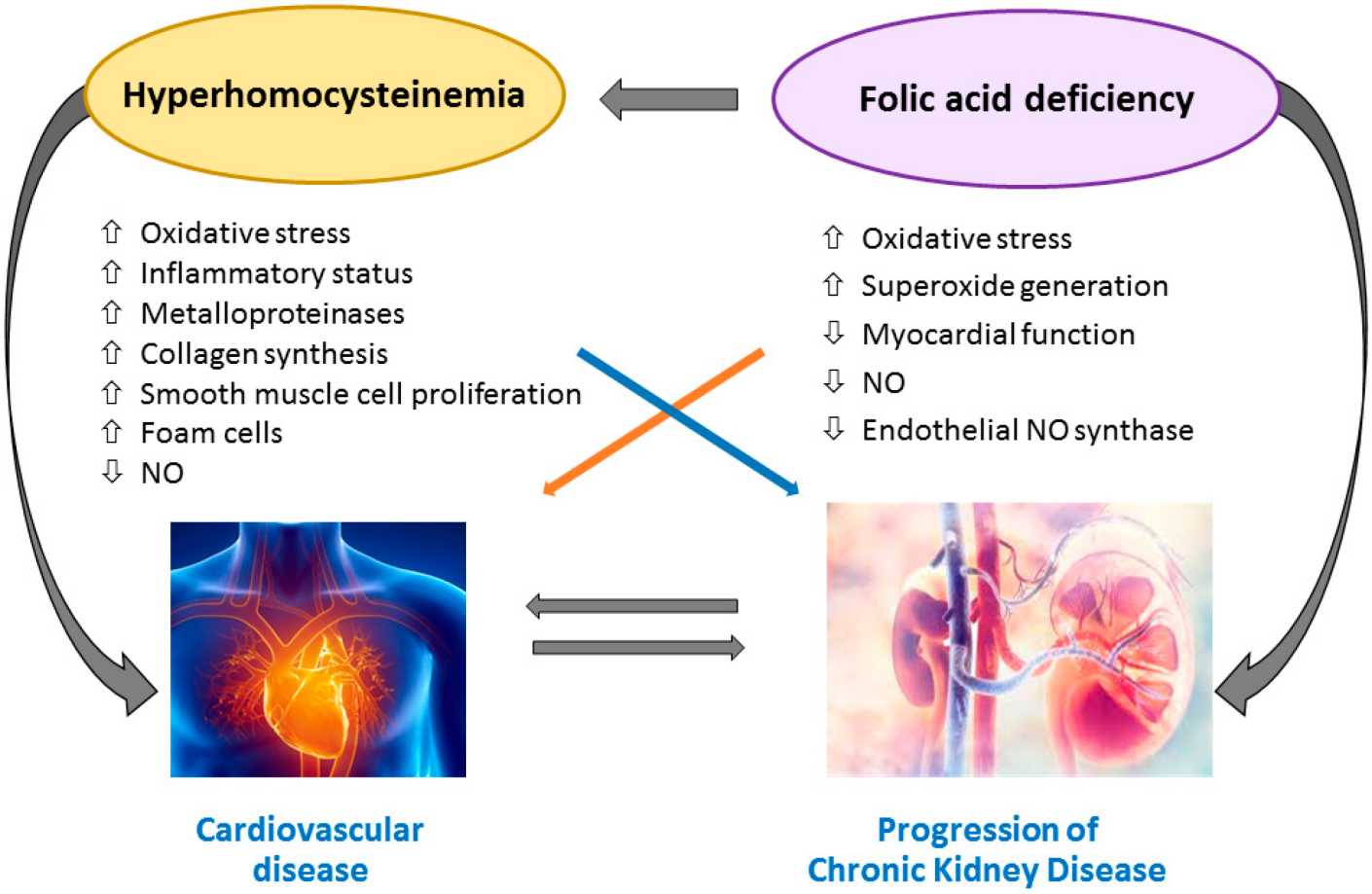

Figure 2. Hyperhomocysteinemia-induced amplification of atherosclerosis and inflammation in chronic kidney disease (CKD) patients. Abbreviations: NO, Nitric Oxide.

6. Effect of Folic Acid and Vitamin B12 Supplementation on CVD and Mortality in CKD and ESRD

Regarding folic acid and vitamin B12 supplementation, the role such vitamins administration with the aim of reducing mortality and prevent progression to ESRD is still to be determined.

Moreover, effective folic acid and vitamin B12 supplementation dosages are not clearly established in the category of patients that take dosages ranging from 2.5 to 5 mg of folic acid three times a week up to more than 15 mg/day. Simultaneous administration of intravenous B complex vitamins is proven to be more efficient in reducing homocysteine serum levels and restoring the remethylation pathway in ESRD patients [72].

Righetti et al. in a one-year, placebo-controlled, non-blinded randomized control trial on a cohort of 81 chronic hemodialysis patients, showed no survival benefit of treatment with folic acid compared to placebo, and only 12% of patients on treatment reached normal homocysteine blood levels [73]. Wrone et al. found no difference in terms of mortality and cardiovascular events in a multicentre study on 510 patients on chronic dialysis randomized to 1, 5, or 15 mg/day of folic acid [74].

In the ASFAST study (Cardiovascular Morbidity and Mortality in the Atherosclerosis and Folic Acid Supplementation Trial), a double blinded, placebo controlled trail, a randomized cohort of 315 CKD dialysis patients (with eGFR < 25 mL/min) were treated with folic acid 15 mg/die or placebo. After a median follow-up of 3.6 years, the results failed to demonstrate a benefit of folic acid therapy regarding all-cause mortality, cardiovascular mortality and control of atheroma progression (carotid intima-media thickness progression) [75].

The HOST trial (Homocysteinemia in Kidney and End Stage Renal Disease) is a double blind, placebo-controlled trial in which 2056 patients with advanced CKD or ESRD requiring renal replacement therapy and elevated homocysteine levels, were randomized to a combined therapy with folic acid, vitamin B12 and piridoxin or placebo. After a median follow-up of 3.2 years, the study showed a significant reduction in homocysteine levels, but failed to reach its primary end-point, reduction of all-cause mortality, and its secondary end-point, reduction in cardiovascular death, amputation and thrombosis of the vascular access. A possible explanation for these negative results may be ascribed to the high cardiovascular comorbidity burden and the suboptimal compliance to therapy. Moreover, the study considered CKD and ESRD population together and was underpowered to evaluate the two populations separately. The disparity between these findings and the previously reported epidemiologic data could reflect limitations of observational studies [76].

Recently, Heinz et al. designed a multicenter trial on 650 chronic hemodialysis patients randomized to 5 mg folic acid, 50 µg vitamin B12 and 20 mg vitamin B6 versus placebo three times a week (post-dialysis) for 2 years. No differences were observed between the two groups in terms of all cause-mortality and fatal and non-fatal cardiovascular events. On the other side, post-hoc analysis revealed a significant reduction in unstable angina pectoris and fewer vascularization procedures [77].

In a meta-analysis by Heinz et al. involving five intervention trials for a total of 1642 dialysis patients treated with folic acid, vitamin B12 and vitamin B6, a significant CVD risk reduction but not mortality risk reduction was demonstrated [10]. Another meta-analysis including 3886 patients with ESRD or advanced CKD (creatinine clearance < 30 mL/min) assessed the relationship between folic acid therapy (with or without vitamin B6 and B12) and CVD. Folic acid reduced cardiovascular risk by 15% in ESRD patients with greater benefit in those treated for longer than 24 months and in those from areas with no or partial grain fortification [78].

Finally, many published post-hoc analyses have shown that several factors including age, baseline homocysteine levels, folic acid fortification of grains, B12 status, renal function, comorbidities and medications could be modifiers of folic acid and vitamin B12 effects on cardiovascular risk [12].

Table 1 summarizes the interventional studies investigating the effects of folic acid and vitamin B12 administration on CVD risk, mortality and CKD progression.

Table 1. Interventional trials on the effects of folic acid and vitamin B12 administration and CVD risk, mortality and CKD progression.

Study	Design/Intervention	Participants, *n*	End Point	Follow-up, Years	Results
Xu et al., 2016 [79]	Double blind RCT: enalapril 10 mg versus enalapril 10 mg plus folic acid	15,104 (eGFR ≥ 30 mL/min). No folic acid fortification	CKD progression	4.4	Enalapril plus folic acid delayed CKD progression
House et al., 2010 [80]	Double blind RCT: folic acid 2.5 mg + Vitamin B6 25 mg + Vitamin B12 1 mg versus placebo	238 (diabetic nephropathy with eGFR > 30 mL/min). Folic acid fortification	CKD progression	2.6	Greater GFR decrease and more CVD events in treatment group
Heinz et al., 2010 [77]	Double blind RCT: folic acid 5 mg, vitamin B12 50 µg, vitamin B6 20 mg versus placebo 3 times a week	650 hemodialysis patients	All-cause mortality, cardiovascular events	2	No differences
Mann et al., 2008 [81]	Double blind RCT: folic acid 2.5 mg + vitamin B6 50 mg + vitamin B12 1 mg versus placebo	619 CKD (eGFR <60 mL/min)	All-cause mortality, cardiovascular events	5	No differences
Cianciolo et al., 2008 [11]	Open label randomized trial: 5-MTHF intravenous. three times a week versus folic acid 5 mg oral daily	314 hemodialysis patients	All-cause mortality	4.5	Less mortality risk in 5-MTHF group (independent of homocysteine)
Jamison et al., 2007 [76]	Double blind RCT (HOST): folic acid 40 mg + vitamin B6 100	2056 CKD (eGFR ≤ 30) or hemodialysis (folic acid fortification)	All-cause mortality, CKD progression	3.2	No differences

Study	Design/Intervention	Participants, *n*	End Point	Follow-up, Years	Results
	mg + vitamin B12 2 mg versus placebo				
Vianna et al., 2007 [82]	Double blind RCT: folic acid 5 mg versus placebo	97 hemodialysis patients	Cardiovascular events	2	No differences
Zoungas et al., 2006 [75]	Double blind RCT (ASFAST): folic acid 15 mg versus placebo	315 CKD (eGFR < 25 mL/min), hemodialysis and peritoneal dialysis	Cardiovascular events and mortality	3.6	No differences
Righetti et al., 2006 [83]	Open prospective trial: folic acid 5 mg versus untreated	114 hemodialysis patients	Cardiovascular events	2.4	Folic acid decreases CVD events
Wrone et al., 2004 [74]	Three arms, double blind RCT: folic acid 1 mg or 5 mg or 15 mg	510 hemodialysis patients	Cardiovascular events and mortality	2	No differences
Righetti et al., 2003 [73]	Placebo-controlled, non-blinded RCT: folic acid 5, 15, 25 mg or placebo	81 hemodialysis patients	Cardiovascular mortality	1	No differences

Abbreviations: CKD, Chronic Kidney Disease; CVD, Cardiovascular Disease; eGFR, estimated Glomerular Filtration Rate; RCT, Randomized Clinical Trial.

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