Aus dem Institut für Agrar- und Ernährungswissenschaften
(Geschäftsführender Direktor: Prof. Dr. Reinhold Jahn)

der Naturwissenschaftlichen Fakultät III
(Dekan: Prof. Dr. Peter Wycisk)

der Martin-Luther-Universität Halle-Wittenberg

Hyperhomocysteinemia, Related Vitamins and Mortality in Patients with End-stage Renal Disease: Results of Observational Investigations and a Randomized Controlled Trial.

Dissertation

zur Erlangung des akademischen Grades
Doktor der Trophologie (Dr. troph.)

von
Diplom-Ökotrophologin Judith Heinz

Halle (Saale) 2009

Aus dem Institut für Agrar- und Ernährungswissenschaften
(Geschäftsführender Direktor: Prof. Dr. Reinhold Jahn)

der Naturwissenschaftlichen Fakultät III
(Dekan: Prof. Dr. Peter Wycisk)

der Martin-Luther-Universität Halle-Wittenberg

Hyperhomocysteinemia, Related Vitamins and Mortality in Patients with End-stage Renal Disease: Results of Observational Investigations and a Randomized Controlled Trial.

Dissertation

zur Erlangung des akademischen Grades
Doktor der Trophologie (Dr. troph.)

vorgelegt von
Diplom-Ökotrophologin Judith Heinz
geb. am 07.07.1978 in Sangerhausen
Gutachter:
Prof. Dr. Gabriele Stangl
Priv.-Doz. Dr. Jutta Dierkes
Prof. Dr. Claus Luley
Verteidigung am: 11.01.2010

Halle/Saale 2010

Danksagung

Da diese Arbeit nur durch die Unterstützung und Mithilfe vieler Menschen möglich wurde, möchte ich all jenen an dieser Stelle danken:

Mein wohl größter Dank gilt PD Dr. Jutta Dierkes, die mich während meiner gesamten Promotionszeit umfassend betreut, unterstützt und gefördert hat. Ich danke ihr für all die zahlreichen motivierenden Gespräche und ebenso für die konstruktive und faire Kritik im Rahmen meiner Doktorarbeit. Die Zusammenarbeit mit ihr hat mir sehr viel Freude bereitet und war eine große Bereicherung für mich.

Herrn Prof. Dr. Claus Luley danke ich dafür, dass er mir das Thema dieser Promotionsarbeit überlassen hat. Ich danke ihm für seine fachliche Unterstützung, Diskussionsbereitschaft und sein Vertrauen, welches er mir entgegen gebracht hat.

Frau Prof. Dr. Gabriele Stangl möchte ich meinen Dank für ihre Bereitschaft zur Betreuung und Unterstützung bei der Einreichung dieser Arbeit aussprechen.

Gleichzeitig bedanke ich mich bei allen Mitarbeitern des Instituts für Klinische Chemie und Pathobiochemie der Otto-von-Guericke Universität Magdeburg, insbesondere bei Steffi Aronica, Dr. Katrin Borucki, Dr. Lilli Wiens und Alexandra Blaik deren ständige Hilfsbereitschaft, Unterstützung und vor allem Freundschaft ganz wesentlich zur Anfertigung dieser Arbeit beigetragen haben. PD Dr. Sabine Westphal danke ich für die unermüdliche Beantwortung medizinischer Fragestellungen und Unterstützung im Laborbereich. Für die zuverlässigen Bestimmungen der Laborparameter möchte ich mich bei allen medizinisch technischen Assistentinnen des Instituts recht herzlich bedanken.

Ein weiterer Dank gilt Dr. Ute Domröse und Prof. Dr. Klaus H. Neumann aus der Klinik für Nephrologie, die jederzeit wichtige Ansprechpartner für alle nephrologischen Fragen und eine große Hilfe bei der Patientenrekrutierung waren. In diesem Zusammenhang möchte ich mich auch bei allen Patienten für die Teilnahme an der Interventionsstudie und bei allen Dialyseärzten sowie ihren Mitarbeitern für die Unterstützung in den Dialysezentren bedanken. Den Mitarbeiterinnen der Zentralapotheke Christina Grabau und Kerstin Weis danke ich für die Logistik der Studienmedikation.

Herrn Prof. Dr. Siegfried Kropf danke ich für eine sehr zuverlässige und angenehme Zusammenarbeit, für die Beratung bei den statistischen Analysen dieser Arbeit und für die unermüdliche Hilfsbereitschaft bei allen auftretenden statistischen Herausforderungen.

Für das Korrekturlesen meiner Promotionsarbeit und die wertvollen stilistischen Verbesserungsvorschläge danke ich Anne und Michael Wegwitz.

Ein ganz besonderer Dank gilt Florian Wegwitz für das stetige Verständnis, das ich von seiner Seite erfahren habe, für die ständige Diskussionsbereitschaft, Geduld und Unterstützung während meiner Promotion.

Meiner gesamten Familie und meinen Freunden danke ich für die persönliche Unterstützung, die sie mir entgegen gebracht haben und Ihr Interesse an meiner Arbeit.

Hyperhomocysteinemia, Related Vitamins and Mortality in Patients with End-stage Renal Disease: Results of Observational Investigations and a Randomized Controlled Trial.

Content		Page
Chapter 1	General introduction	1
Chapter 2 *	Homocysteine as a Risk Factor for Cardiovascular Disease in Patients Treated by Dialysis: A Meta-Analysis.	34
Chapter 3	Washout of Water-Soluble Vitamins and of Homocysteine during Haemodialysis – Effect of High-Flux and Low-Flux Dialyzer Membranes.	58
Chapter 4	Vitamins are Associated with Survival in Patients with End-stage Renal Disease: a 4-year Prospective Study.	72
Chapter 5	Influence of a Supplementation with Vitamins on Cardiovascular Morbidity and Mortality in Patients with End-stage Renal Disease: Design and Baseline Data of a Randomized Clinical Trial	89
Chapter 6 *	B-Vitamins and the Risk for total Mortality and Cardiovascular Disease in End-stage Renal Disease. Results of a Randomized Controlled Trial.	94
Chapter 7	Overall Discussion and Conclusion	112
Summary of the thesis		124
Zusammenfassung		127
Annex		I
List of publications and presentations		II
Curriculum vitae		V

** For reasons of copyright, original articles are only included in abstract-form in the published version of this dissertation. The original articles are available from the respective journals.*

1

General Introduction

Homocysteine

- Chemistry and metabolism
- Homocysteine related vitamins

Hyperhomocysteinemia

- Environmental factors
- Genetic modulation
- Homocystinuria

Homocysteine and atherosclerotic disease

- Association between homocysteine and cardiovascular disease
- Homocysteine toxicity and pathophysiological mechanisms
- Effects of B vitamins on homocysteine
- Effects of B vitamins on total mortality and cardiovascular disease

Patients with chronic kidney disease (CKD)

- Renal pathophysiology
- CKD and risk for cardiovascular disease and mortality
- Traditional risk factors in patients with CKD
- Non-traditional risk factors in patients with CKD

Homocysteine in patients with chronic kidney disease

- Hyperhomocysteinemia in patients with CKD
- Homocysteine and risk for cardiovascular disease and mortality in patients with CKD
- B vitamins in patients with CKD
- Effects of B vitamins on total mortality and cardiovascular disease in patients with CKD

Scope of the thesis

References

Abbreviations:

AdoMet	S-adenosylmethionine
AdoHcy	S-adenosylhomocysteine
DNA	deoxyribonucleic acid
RNA	ribonucleic acid
tHcy	Total homocysteine (sum of free and protein-bound homocysteine)
CBS	Cystathionine-β-synthase
THF	Tetrahydrofolate
MTHFR	Methylenetetrahydrofolate reductase
PLP	Pyridoxal-5'-phosphate
BHMT	Betaine-homocysteine methyltransferase
AHA	American Heart Association
MSR	Methionine synthase reductase
SHMT	Serine hydroxymethyltransferase
TC	Transcobalamin
TS	Thymidylate synthase
COMT	Catecholamine-O-Methyltransferase
GCPII	Glutamate carboxypeptidase II
RFC	Reduced folate carrier
ROS	Reactive oxygen species
LDL	Low density lipoprotein
RCT	Randomized controlled trial
CHAOS-2	Second Cambridge Heart Antioxidant Study
VISP	Vitamin Intervention for Stroke Prevention
NORVIT	Norwegian Vitamin Trial
WENBIT	Western Norway B-Vitamin Intervention Trial
HOPE-2	Heart Outcomes Prevention Evaluation-2
WAFACS	Women's Antioxidant and Folic Acid Cardiovascular Study
SEARCH	Study of Effectiveness of Additional Reductions in Cholesterol and Homocysteine
RR	Relative risk
FA	Folic acid
CKD	Chronic kidney disease

BUN	Blood urea nitrogen
GFR	Glomerular filtration rate
MDRD	Modification of Diet in Renal Disease
SUN	Serum urea nitrogen concentration
ESRD	End-stage renal disease
HD	Haemodialysis
PD	Peritoneal dialysis
MICS	Malnutrition-inflammation complex syndrome
DOPPS	Dialysis Outcomes and Practice Patterns Study
HOST	Homocysteinemia in Kidney and End Stage Renal disease
ASFAST	Atherosclerosis and Folic Acid Supplementation Trial

Homocysteine

Chemistry and metabolism

The sulfur-containing, non-proteinogenic amino-acid homocysteine is formed by demethylation of the essential amino acid methionine. The intermediate compounds of this reaction are S-adenosylmethionine (AdoMet) and S-adenosylhomocysteine (AdoHcy). AdoMet is a methyl donor in numerous different cellular methyltransferase reactions, including those of DNA, RNA and proteins.[1]

In plasma, around 70% of homocysteine is bound to proteins by disulfide bridges. A smaller, non-protein bound fraction of homocysteine consists of homocystine, the disulfide of homocysteine, and of mixed disulfides of homocysteine with cysteine. Only 1-2% is reduced homocysteine, also referred to as 'free' homocysteine (Figure 1).[2] In the literature all homocysteine fractions are summarized as 'total plasma homocysteine' and conventionally abbreviated with 'tHcy'. Homocysteine exists in D- and L-enantiomers, whereas L-homocysteine is the physiologically relevant form.

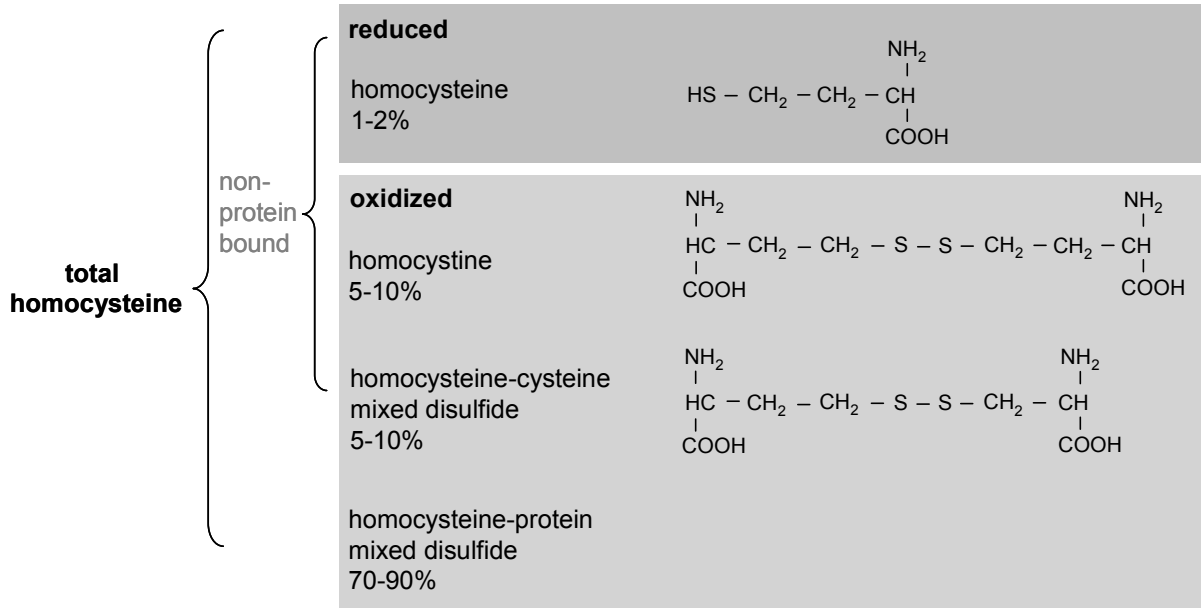

Figure 1. Forms of homocysteine and distribution in the plasma

Homocysteine is metabolized via two different pathways: the transmethylation pathway and the transulfuration pathway (Figure 2). The irreversible transsulfuration pathway is mainly limited to cells of the liver and the kidney. Homocysteine is degraded to cysteine involving the enzymes cystathionine-β-synthase (CBS) and γ-cystathionase, both dependent on

pyridoxal-5'-phosphate, a biologically active form of vitamin B6, as cofactor.[3] The transmethylation pathway is a folate- and vitamin B12-dependent reaction. Homocysteine is remethylated to methionine by the enzyme methionine synthase (MS), which uses methylcobalamin, a biologically active form of vitamin B12. The methyl-donor in this reaction is 5-methyl-tetrahydrofolate (5-methyl-THF), which is synthesized from tetrahydrofolate via 5,10-methylenetetrahydrofolate (5,10-methylen-THF) by the enzyme 5,10-methylenetetrahydrofolate reductase (MTHFR). In certain tissues, such as kidney and liver, the alternative, folate non-dependent methyl-donor is betaine under participation of betaine-homocysteine methyltransferase.[4]

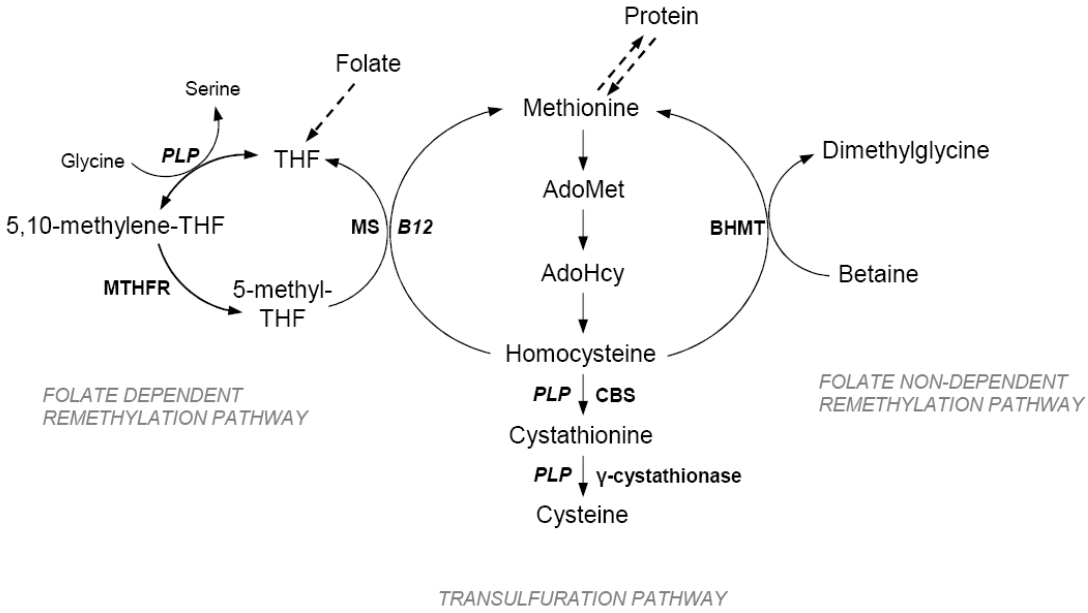

Figure 2. Simplified homocysteine metabolism. AdoMet, S-adenosylmethionine; AdoHcy, S-adenosylhomocysteine; CBS, Cystathionine-β-synthase; BHMT, Betaine-homocysteine methyltransferase; MS, Methionine synthase; PLP, pyridoxal-5'-phosphate; THF, Tetrahydrofolate; B12, Vitamin B12

Homocysteine related vitamins

The water-soluble vitamins folic acid, vitamin B12 and B6 are involved in the metabolism of homocysteine.

Regarding the occurrence of **folic acid,** different terms are used to denote this vitamin. The synthetic form of the vitamin is called folic acid and the naturally occurring forms are summarized as folate (food folate). The primary chemical forms of dietary folate are polyglutamates, which must be deconjugated by hydrolysis to the monoglutamyl form before being absorbed in the intestinal tract.[5] In the liver non-methylated folate is transformed into

the methylated form. The main circulating form of folate in the blood is 5-methyl-THF, which is transferred across the cell membranes via a specialized membrane carrier system.[6] In the cells 5-methyl-THF is demethylated and converted back into polyglutamates. The demethylation process is the interface with the homocysteine metabolism. Besides its role in the methylation of homocysteine to methionine, folate is important for the synthesis of purines and pyrimidines that are incorporated into DNA and RNA. Folate is furthermore involved in the transformation of serine to glycine as well as in the metabolism of histidine and tryptophan.[7]

Natural rich sources of folate are dark green leafy vegetables, oranges, tomatoes, liver and cereals. The bioavailability estimates for folate range from approximately 50% for naturally occurring food folate to 100% for synthetic folic acid.[5,8] Synthetic folic acid is used for supplements and food fortification. Grain fortification was mandated in countries, such as the United States, Canada, Chile, and Hungary, to increase the folic acid intake in women of childbearing age in order to reduce the risk of severe malformations, as neural tube defects.[9,10] Adequate folate status has been defined as a serum or plasma concentration of folate above 3ng/mL (6.8nmol/L) and red blood cell folate concentration above 140ng/mL (317nmol/L).[10] The recommendation of the German Nutrition Society for healthy adults is a daily folate intake of 400µg.[11] Even if toxic effects of folic acid in high amounts (15mg/day) were not observed, high dose folic acid intake can mask a sub clinical vitamin B12 deficiency. Haematological symptoms due to the vitamin B12 deficiency, e.g. megaloblastic anaemia, are compensated by folic acid, but the neurological component of the vitamin B12 deficiency persists.

The active form of **vitamin B12**, methylcobalamin, is also involved in the methylation of homocysteine to methionine. Vitamin B12 is only found in animal products, like meat, fish, eggs and milk. The recommended daily intake is 3µg.[11] Although common Western diets provide an adequate vitamin B12 intake, a deficiency of this vitamin occurs frequently (>20%) among elderly people.[12] More than a half of these deficiencies are due to food-cobalamin malabsorption caused by gastrointestinal problems, which are characterized by the inability to release cobalamin from food or a deficiency of intestinal cobalamin transport proteins or both. A vitamin B12 deficiency can lead to severe neurologic damage and life-threatening anaemia; therefore, such individuals require medical treatment including vitamin B12 injections.[13]

The metabolically active form of **vitamin B6** is pyridoxal-5'-phosphate and is an important co-enzyme in the metabolism of amino acids. Altogether, vitamin B6 participates in more

than 100 enzymatic reactions and is needed amongst others for the transformation of tryptophan to niacin and for the formation of neurotransmitters. It is involved in the transsulfuration of homocysteine to cystathionine and cysteine as well as in the folate metabolism, since it is coenzyme in the transformation of glycine to serin. Dietary sources for vitamin B6 are meat, fish, legumes, nuts and cereals. Deficiencies, which can result in cheilosis, stomatitis and effects on the central nervous system, are uncommon.[14]

Hyperhomocysteinemia

The definition of the ranges of normal homocysteine plasma level is not standardized at present, as there are numerous factors influencing the physiological homocysteine concentrations. Therefore, considerable differences exist in reference levels used. According to the American Heart Association (AHA) advisory statement, elevated plasma homocysteine concentrations are classified as moderate (16-30µmol/L), intermediate (31-100µmol/L), and severe (greater than 100µmol/L). Hyperhomocysteinemia is often defined as homocysteine concentrations in the 90th or 95th percentile of a control population, which is in most of the studies approx. 15µmol/L.[15] Hyperhomocysteinemia refers to a condition of increased circulating concentrations of total homocysteine in the plasma due to possible disturbances in the intracellular homocysteine metabolism.

Environmental factors

As the **B-vitamins** folate, cobalamin and pyridioxal-5'-phosphate are involved in the metabolism of homocysteine, a B-vitamin deficiency is probably the most common cause of moderate hyperhomocysteinemia.[16]

Increasing **age and male sex** are associated with higher homocysteine concentrations.[17,18] Many studies have shown that homocysteine levels increase with age and hyperhomocysteinemia is very common with the oldest age subjects.[19,20] Decline of renal function and inadequate vitamin intake may explain the increased homocysteine levels observed in older age. Differences between males and females may be explained by sex hormones and larger muscle mass in men, since the formation of muscles is associated with the simultaneous formation of homocysteine in connection with creatine, the precursor of creatinine synthesis.[21,22]

Different **diseases** influence homocysteine concentration. The most frequent clinical cause of hyperhomocysteinemia is renal failure. However, the underlying mechanism for the elevated homocysteine level in these patients is not completely clear and will be specified in a later section. Intestinal disorders like ulcerative colitis and Crohn's disease may lead to a deficiency of folate or vitamin B12 and may increase homocysteine in plasma.[23]

Lifestyle factors, too, are determinants of the homocysteine concentration. Smoking is positively associated with plasma homocysteine,[24,25] however, because of a generally less healthy diet of smokers, residual confounding due to the B-vitamin intake cannot fully be excluded.[26] Between homocysteine levels and alcohol consumption a J-shaped association was observed.[27] Thus, moderate alcohol consumers have lower homocysteine concentrations than non drinkers. In alcoholics, elevated homocysteine levels were observed, possibly due to disturbances in the vitamin metabolism caused by gastrointestinal disturbances and liver damage.[28,29] However, not all studies confirmed the relationship between alcohol and homocysteine.[30] Another homocysteine influencing lifestyle factor is coffee consumption.[31] One reason for the increase of homocysteine may be caffeine, which inhibits the conversion of homocysteine to cysteine by acting as a vitamin B6 antagonist.[32] Another possible reason may be the high intake of polyphenols. By methylation of polyphenols a methyl group is transferred from S-adenosylmethionine to polyphenols under formation of homocysteine.[33]

Additionally, fasting status or rather the amount of time since last meal influences homocysteine plasma concentrations. The plasma level is generally higher with increasing time since last meal, however, the difference between fasting and nonfasting homocysteine levels is low (approx. 0.5µmol/L).[34]

Several **drugs** have influence on homocysteine level, for example antiepileptic drugs, methotrexate, and lipid-lowering drugs. Antiepileptic drugs influence the folate metabolism through inhibition of enzymes involved in the metabolism and lead to increased homocysteine concentrations.[35] Methotrexate, an antifolate, is used for the treatment of cancer and autoimmune diseases because of its inhibition of cell reproduction.[36] Lipid-lowering drugs like fibrates and niacin indeed reduce blood lipids, but have an elevating effect on homocysteine levels.[37,38]

Genetic modulation

The homocysteine plasma concentration is not only influenced by environmental factors but also by genetic variations. Numerous variations in genes involved in homocysteine or vitamin

metabolism have already been described (Table 1). The most investigated polymorphism is the 677C>T transition in the MTHFR gene, yielding a thermolabile variant of the enzyme with decreased activity. The remethylation of homocysteine to methionine is disturbed (Figure 2).[39] In the general population the prevalence of homozygosity is 5 to 15% in most Caucasian populations.[3] Subjects who are homozygous for the 677T-allele tend to have mildly increased blood concentrations of homocysteine by approx. 25%, but only if their folate intake is insufficient.[40] If the folate status is normal, the inactivation of the enzyme may be overcome and homocysteine levels are not affected.[41] The effect on homocysteine level of the other variations is less clear then observed for the C677T MTHFR polymorphism (Table 1).

Table 1: Genetic variants in genes involved in the homocysteine or vitamin metabolism and the effect on homocysteine level (+ clear association; ± possible association; 0 no association)

Gene	Genetic variant	Amino acid substitution	Effect on homocysteine level
MTHFR [39,42]	C677T	A226V	+ (25%)
	A1298C	E433A	0
CBS [42,43]	844ins68	-	0
	14037 31 bp VNTR	-	+
	C699T	Y233Y	0
	C1080T	A360A	0
MS [42]	A2756G	D919G	0
MSR [42]	A66G	I22M	0
BHMT [43]	G595A	G199S	0
	G716A	Q239R	0
	G1218T	Q406H	0
SHMT [44]	C1420T	L474F	±
TC [43,45,46]	A67G	I23V	±
	G280A	G94S	0
	C776G	P259R	±
	C1043T	S348F	0
	G1196A	R399Q	0
TS [47]	2R/3R	-	±
COMT [48]	G1947A	V158M	±
GCPII [49]	C1561T	H475Y	±
RFC [50]	G80A	R27H	±

Homocystinuria

A more severe, but also a less frequently form of hyperhomocysteinemia is the recessively inherited disorder of metabolism 'homocystinuria' caused by a deficiency of the vitamin B6-dependent enzyme CBS. Enzymes of the folate or cobalamin metabolism can be affected as well, but this occurs less frequently. The worldwide birth prevalence based on newborn screening has been reported at 1 in 344 000, a minimal incidence because cases are known to be missed by such screening.[51] There is a much higher birth prevalence for example in Ireland, estimated at 1 in 65 000, derived from newborn screening and clinically detected cases.[52] The two most common CBS mutations are the vitamin B6 responsive mutation I278T and the vitamin B6 non-responsive mutation G307S. A block at CBS limits transsulfuration and results in increased homocysteine in plasma or urine and increased methionine, the latter caused by enhanced remethylation.[53,54] The homocysteine plasma levels are at least 10 times higher than in the general population, between 100 and 300µmol/L.[55]

Independently of the specific enzymatic abnormality which leads to hyperhomocystinuria, this abnormality affects the eye, skeletal system, vascular system, and central nervous system.[54] The major consequences are ectopia lentis, osteoporosis, mental retardation, pathological vascular lesions and thromboembolic events already in the first years of life. Thromboembolism is the major cause of morbidity and early death in these patients and can affect any vessel.[56] The observation of the association between homocystinuria and atherosclerotic plaques in vessels of affected children has led to the suggestion that the amino acid homocysteine may be the reason for the atherosclerotic changes in patients with hyperhomocysteinemia.[57] This assumption was the basis for the 'homocysteine hypothesis' that also moderate elevated homocysteine concentrations may be a risk factor for atherosclerosis in the general population and yielded to several studies on homocysteine and cardiovascular diseases.

In vitamin B6 responsive patients with homocystinuria, mega doses of vitamin B6 delay or prevent premature vascular injury, even if, despite treatment, the homocysteine concentrations of these patients are above the normal range.[56]

Homocysteine and atherosclerotic disease

The results of many observational studies added to the 'homocysteine hypothesis' and reported that patients with coronary heart disease or stroke have higher homocysteine blood concentrations than controls.

Association between homocysteine and cardiovascular disease

Starting in the 1970s, the association between homocysteine and cardiovascular disease has been studied in small case-control studies. The first meta-analysis of the observational studies on this topic in 1995 indicated that an increase of homocysteine about 5µmol/L is associated with a 70% higher risk for coronary heart disease.[58] However, the included studies were mainly retrospective ones and it was not possible to exclude a reverse causality bias, which means that the atherosclerotic disease might itself have increased homocysteine concentrations. An updated meta-analysis in 1998 reported a weaker association in prospective studies than in retrospective studies. In the prospective studies, a 5µmol/L higher homocysteine level was associated only with a 30% increased risk for coronary heart disease.[59] In 2002, the Homocysteine Studies Collaboration carried out a meta-analysis based on individual patients' data, which allowed adjusting for established cardiovascular risk factors like age, sex and cholesterol as examples. Homocysteine decrease by 25% was associated with an 11% lower risk for coronary heart disease and a 19% lower risk for stroke.[60] A meta-analysis, which also took into account the influence of the genotype of the MTHFR gene on the association between homocysteine and vascular disease, yielded similar results and provided strong support for a causal relationship.[61]
However, even if this association is known since several decades the underlying mechanism of homocysteine in the development of atherosclerotic diseases is highly discussed and cannot be clearly answered to date.

Homocysteine toxicity and pathophysiological mechanisms

There are many theories by which increased homocysteine concentration may contribute to atherosclerotic disease.

Creation of a prothrombotic environment

Although platelets have normal life span and morphology in patients with hyperhomocysteinemia, homocysteine may activate platelets, increases platelet aggregation and adhesion. Furthermore, it was observed that homocysteine acts on the coagulation cascade and on fibrinolyses.[62] Therefore, homocysteine disturbs the procoagulant-anticoagulant balance and creates directly or in interaction with other risk factors a prothrombotic environment.[63]

Toxicity mechanisms - oxidation, nitrosylation, homocysteinylation, and hypomethylation

Homocysteine can react as a pro-oxidant and is involved in the production of hydrogen peroxide and, in the presence of nitric oxide, in the formation of the powerful oxidant peroxynitrite. The formation of ROS (reactive oxygen species) may lead to lipid peroxidation, which initiates an inflammatory response and may lead to atherosclerotic lesions.[64] Also if the results of the studies are not consistent regarding the LDL-oxidation promoting effects of homocysteine,[65] a strong correlation between homocysteine and the activity of extracellular superoxide dismutase, which is an important antioxidant in vascular tissue, was found in patients with hyperhomocysteinuria. This may be an indicator for a protective antioxidant response to homocysteine-induced oxidative damage.[66]

Another possible protective mechanism against the adverse effects of homocysteine is the formation of S-nitroso-homocysteine by oxidation of homocysteine with nitric oxide. S-nitroso-homocysteine has vasodilatory and platelet anti-aggregation properties. However, a chronic exposure to high homocysteine concentrations may lead to a reduced production and bioavailability of nitric oxide and to oxidative injury.[64,67]
Homocysteine may be furthermore responsible for the inhibition of the expression of the enzyme endothelial glutathione peroxidase, which catalyzes the reduction of hydrogen and lipid peroxides and prevents the oxidative inactivation of nitric oxide.[67,68]

One of the main mediators of homocysteine toxicity is the homocysteinylation of proteins leading to structural and functional alterations at the molecular and cellular level. Protein-homocysteinylation is the post-biosynthetic acylation of free amino groups in proteins by

homocysteine thiolactone. Consequences are protein damage with altered electrophoretic mobility and loss of enzymatic activity due to protein denaturation.[69]

Recent publications discuss epigenetic mechanisms in the relationship between homocysteine and atherogenesis. Atherogenesis is believed to involve mainly functional gene alterations resulting from alterations in the gene expression or from changes in gene product activity.[70] Experimental studies suggest a link between homocysteine, and its intermediates, and altered methylation products.[70] Homocysteine can regulate methylation reactions by the transmethylation reaction from the methyl donor AdoMet to AdoHcy. A homocysteine accumulation leads to accumulation of AdoHcy, which is a strong inhibitor of most AdoMet-dependent methyltransferases and is associated with DNA hypomethylation.[71-73]

Influence on endothelial function

In vitro it was observed that homocysteine enhances vascular smooth muscle cell proliferation by the induction of cyclin A gene expression and increased transcription of cyclin-dependent kinase, which is a regulatory protein in mitosis.[74]
In vivo it was observed that hyperhomocysteinemia may alter vascular morphology and influence the endothelial function. Endothelial dysfunction is assumed to be an early sign of vascular damage. The endothelium-dependent vasodilation is utilized as a non-invasive method to measure endothelial function and therefore to detect pre-atherosclerotic alterations. In several studies in hyperhomocysteinemic patients or healthy subjects after a methionine load a homocysteine induced reduction in endothelium-dependent, flow-mediated vasodilation was observed.[75,76] *In vitro*, homocysteine can impair the production or bioavailibility of vasoactive mediators like endothelin-1, prostacyclin, and nitric oxide, which are important for the maintenance of vascular haemostasis.[77,78]

Effects of B vitamins on homocysteine

Many studies demonstrated that supplementation with B-vitamins can be used as a simple, safe and inexpensive therapy to lower homocysteine levels. It was summarized that in the general population the intake of folic acid supplements at a dosage of 0.5 to 5mg per day is associated with a reduction in homocysteine plasma levels by about 25%. The addition of vitamin B12 lowers homocysteine concentrations by a further 7%. The addition of vitamin B6

to folic acid supplements was not associated with any further reduction in plasma homocysteine levels.[79]

Effects of B vitamins on total mortality and cardiovascular disease

Given that it is possible to reduce homocysteine concentration by folic acid and vitamin B12 it was hypothesized that a supplementation with these vitamins may reduce the risk for cardiovascular disease and mortality. Starting in the mid-1990s, a number of trials with B vitamins were initiated to test this hypothesis. The first results of randomized studies in patients with coronary angioplasty were promising since a reduction of about 40% of the risk for restenosis or revascularization was observed after supplementation with folic acid, vitamin B12 and B6.[80,81] However, the recently published large-scale, randomized controlled trials (RCTs) in a high risk population for cardiovascular disease, as for example the Second Cambridge Heart Antioxidant Study (CHAOS-2), the Vitamin Intervention for Stroke Prevention (VISP), the Norwegian Vitamin Trial (NORVIT), the Western Norway B-Vitamin Intervention Trial (WENBIT), the Heart Outcomes Prevention Evaluation-2 (HOPE-2), the Women's Antioxidant and Folic Acid Cardiovascular Study (WAFACS) and the Study of Effectiveness of Additional Reductions in Cholesterol and Homocysteine (SEARCH) have so far failed to demonstrate significant reductions in different primary outcome variables (Table 2).[82-88] The results of the HOPE-2 study indicated a significant risk reduction in stroke of about 25%, which was a secondary outcome in this study.

A first meta-analysis, published in 2006, which was carried out to review the results of RCTs, yielded a summary relative risk (RR) of 1.04; 95% CI, 0.92-1.17 for coronary heart disease and of 0.86; 95% CI, 0.71-1.04 for stroke.[89] In a further meta-analysis randomized studies with stroke as endpoint were summarized.[90] This analysis showed a pooled risk reduction of approx. 18% (p=0.045), which was even greater in studies with a follow up period of more than 36 months (RR 0.71; 95% CI, 0.57-0.87; p=0.001).

Table 2: Large-scale randomized controlled trials on B vitamins and cardiovascular disease and total mortality

Trial	Patients	Primary outcome	Treatment *	Treatment effects RR (95%CI)
CHAOS-2 [82]	patients with ischaemic heart disease n = 1882	myocardial infarction (nonfatal), cardiovascular mortality, revascularisation procedures	FA: 5mg vs. placebo	combined endpoint 0.97 (0.72-1.29)
VISP [83]	patients with history of stroke n = 3680	recurrence stroke	FA: 2.5mg; B12: 0.4mg; B6: 25mg vs. FA: 20µg; B12: 6µg; B6: 200µg	stroke 1.0 (0.8-1.3)
NORVIT [84]	patients with myocardial infarction n = 3749	recurrence myocardial infarction, stroke, sudden cardiac death	(A) FA: 0.8mg; B12: 0.4mg; B6: 40mg or (B) FA: 0.8mg; B12: 0.4mg or (C) B6: 40mg vs. placebo	combined endpoint (A) 1.22 (1.0-1.5) (B) 1.08 (0.93-1.25) (C) 1.14 (0.98-1.32)
WENBIT [85]	patients with coronary angiography n = 3096	total mortality, myocardial infarction (nonfatal), unstable Angina pectoris, stroke (nonfatal)	(A) FA: 0.8mg; B12: 0.4mg; B6: 40mg or (B) FA: 0.8mg; B12: 0.4mg or (C) B6: 40mg vs. placebo	combined endpoints (A) and (B) vs. (C) and placebo 1.09 (0.90-1.32) (A) and (C) vs. (B) and placebo 0.90 (0.74-1.09)
HOPE-2 [86]	patients with cardiovascular disease or diabetes mellitus n = 5522	cardiovascular mortality, myocardial infarction, stroke	FA: 2.5mg; B12: 1mg; B6: 50mg vs. placebo	combined endpoint 0.95 (0.84-1.07)
WAFACS [87]	women with cardiovascular disease or at high risk for cardiovascular disease n = 5442	myocardial infarction, stroke, revascularisation procedures, cardiovascular mortality	FA: 2.5mg; B12: 1mg; B6: 50mg vs. placebo	combined endpoint 1.03 (0.90-1.19)
SEARCH [88]	patients with myocardial infarction n = 12064	myocardial infarction (nonfatal), coronary death, revascularisation procedures, stroke	FA: 2mg; B12: 1mg vs. placebo	combined endpoint (without stroke) 1.04 (0.97-1.12)

* FA=folic acid

Patients with chronic kidney disease (CKD)

Renal pathophysiology

Chronic kidney disease (CKD) is a clinical syndrome arising from irreversible, usually progressive, kidney injury. Normal kidneys assume three main functions: excretion of waste products of nitrogen metabolism, regulation of water and electrolyte balance, and specific endocrine functions. A characteristic phenomenon of renal failure is the elevation of creatinine and blood urea nitrogen (BUN) concentrations in the extracellular fluid caused by a decrease in the glomerular filtration rate (GFR). The other functions of the kidney are also impaired, such as synthesis of renal hormones. A wide range of symptoms, signs, and laboratory alterations accompany various degrees of renal failure.
The GFR is traditionally considered as the best overall index of renal function in health and disease:[91]

$$\text{GFR (mL/min/1.73m}^2\text{)} = \frac{\text{creatinine (urine)} * \text{urine flow rate}}{\text{creatinine (serum)}}$$

(adjusted for a standard body surface area of 1.73 m^2)

Because GFR is difficult to measure in clinical practice, most clinicians estimate the GFR from the serum creatinine concentration by use of formulas. Until recently the most widely used formula was the *Cockroft-Gault* formula that takes creatinine, weight and age into account:[92]

$$\text{GFR (mL/min)} = \frac{(140 - \text{age}) * \text{weight}}{72 * \text{creatinine (serum)}}$$

Recent studies yielded the MDRD (Modification of Diet in Renal Disease) formula to estimate the GFR:[93]

$$\text{GFR (mL/min/1.73m}^2\text{)} = 170 * [\text{creatinine (serum)}]^{-0.999} * [\text{age}]^{-0.176} * [0.762 \text{ if patient is female}] * [1.180 \text{ if patient is black}] * [\text{SUN}]^{-0.170} * [\text{albumin}]^{0.318}$$

(SUN = serum urea nitrogen concentration)

CKD is the general term used to describe irreversible loss of GFR over a prolonged period of time. Chronic renal insufficiency implies mild CKD, whereas the degree of renal failure is not well defined. Uraemia is the symptomatic phase of renal failure during which signs and

symptoms of renal dysfunction are detected. End-stage renal disease (ESRD) refers to any form of CKD at a stage that permanent renal replacement therapy is indicated, such as haemodialysis (HD), peritoneal dialysis (PD) or kidney transplantation.[94]
Chronic kidney disease is classified in 5 stages depending on the severity in kidney damage (Table 1).

Table 3: Stages of chronic kidney disease according to the National Kidney Foundation; GFR is the glomerular filtration rate and is used for measurement of renal function [95]

Stage	Description	GFR; mL/min per 1.73m²
1	Kidney damage with normal or increased GFR	≥90
2	Kidney damage with mildly decreased GFR	60-89
3	Moderately decreased GFR	30-59
4	Severely decreased GFR	15-29
5	Kidney failure (end-stage renal disease)	<15 or dialysis

Renal injury can occur from a variety of diseases, e.g. diabetes mellitus (diabetic nephropathy), hypertension (nephrosclerosis), glomerulonephritis, polycystic kidney disease and chronic interstitial nephritis. In some patients, the underlying cause of kidney failure is unknown.
In 2006, more than 66.500 patients with ESRD were treated with haemodialysis or peritoneal dialysis in Germany. Since 1997 an annual increase of prevalence by 4.4% was observed.[96]

CKD and risk for cardiovascular disease and mortality

Chronic kidney disease is associated with poor prognosis for survival and cardiovascular complications. Patients treated with dialysis have a 5-20fold risk for cardiovascular mortality and a higher prevalence of atherosclerosis compared to the general population.[97] Clinical presentations of atherosclerosis include angina pectoris, myocardial infarction, sudden cardiac death, cerebrovascular disease and peripheral disease.[98]
The risk factors for cardiovascular disease in patients with chronic kidney disease are classified in ‘traditional’ and ‘non-traditional’, where traditional risk factors are those defined in the Framingham Heart Study for the general population, used to predict coronary heart disease outcomes.[99] Non-traditional risk factors are related to the uraemic condition in chronic kidney disease and may explain the excess risk for cardiovascular disease in these patients.[97]
It is unclear whether some of the abnormalities are the cause or the result of the underlying kidney dysfunction and the associated metabolic disturbances.[100]

Traditional risk factors in patients with CKD

As in the general population age and male gender are also traditional risk factors in patients with chronic kidney disease.[100] Lifestyle factors like smoking and physical exercises have not been studied extensively in these patients, however, it can be expected that smoking and physical inactivity enhance the risk for cardiovascular disease.[101,102] Diabetes mellitus, one of the most common causes of ESRD, is at the same time a well-established and important risk factor for cardiovascular disease.[100] Hypertension, too, is a common cause of chronic kidney disease and has been implicated as a major cause of poor clinical outcome and high mortality in dialysis patients.[103] However, several studies demonstrated an inverse association between blood pressure and mortality in dialysis patients. A low predialysis blood pressure was paradoxically associated with high mortality rates and high blood pressure seemed to enhance survival.[104,105] Such inverse associations were also observed for serum cholesterol and the body mass index,[106,107] which are established risk factors in the general population. The 'reverse epidemiology' phenomenon is highly discussed and may explain why efforts to treat conventional risk factors in dialysis patients have not resulted in significant improvement of the poor clinical outcome.[108]

Non-traditional risk factors in patients with CKD

Since traditional risk factors cannot fully explain the high risk status of patients with chronic kidney disease, attention has been focused on non-traditional risk factors. These risk factors include inflammation status, disturbances of mineral metabolism, endothelial dysfunction and oxidative stress.

The malnutrition-inflammation complex syndrome (MICS) is frequently found in patients treated with dialysis and has been implicated as a main cause for the reverse epidemiology and may lead to high risk for cardiovascular disease and mortality.[109] Dialysis patients are exposed to a wide variety of conditions causing inflammation processes. These conditions include amongst others infections of the dialysis access, bioincompatible dialysers and dialysates, as well as acute or chronic illnesses such as ulcers. The inflammatory answer is the release of positive and negative acute-phase reactants. Laboratory parameters such as C-reactive protein, ferritin, interleukin-6 and fibrinogen are therefore elevated in dialysis patients, while albumin and prealbumin as negative acute phase reactants and at the same time marker for malnutrition are decreased during inflammation.[110,111] So far, a treatment of

inflammatory events has not yet been established and the only possibility is the prevention and treatment of sources of chronic infection.[102]

Since high homocysteine levels were identified as potent cardiovascular risk factor in the general population, and given that derangements in the homocysteine metabolism are consistently found in patients with chronic kidney disease, leading to very high homocysteine levels, great attention has also been focused on the role of homocysteine in these patients for the development of cardiovascular disease.

Homocysteine in patients with chronic kidney disease

Hyperhomocysteinemia in patients with CKD

The prevalence of hyperhomocysteinemia in patients with end-stage renal disease is nearly 100% with homocysteine concentrations up to 100µmol/L.[112] The reasons for hyperhomocysteinemia in these patients are, however, unclear. Generally, it is assumed that the reduced kidney function leads to increased homocysteine concentrations in the plasma. However, it can not be fully excluded that hyperhomocysteinemia itself has a negative effect on renal function. Although this question cannot be answered at present, the close correlation between the GFR and homocysteine is assured.[113] Therefore, the conclusion might be obvious that homocysteine is cleared from the body by glomerular filtration and urinary excretion, a process which is disturbed in renal failure. However, only a small fraction of homocysteine circulates in a free form in the human plasma and is available for glomerular filtration. The filtration fraction is only 1% and only very small amounts of homocysteine are normally found in urine.[114] Therefore, decreased urinary excretion does not explain hyperhomocysteinemia in patients with chronic kidney disease. Although homocysteine transsulfuration and remethylation enzymes are present in kidney tissue, studies in humans with normal kidney function found no evidence for an intrarenal homocysteine metabolism, which may be impaired in patients with renal failure.[115]

Another explanation may be alterations in the sulphur amino acid metabolism due to uraemic conditions. Several studies demonstrated that hyperhomocysteinemia can neither be explained by an impaired folate metabolism as a result of renal failure, nor by a higher prevalence of the C677T MTHFR gene polymorphism.[116,117] However, the plasma levels of cystathionine, cysteine, S-adenosylmethionine and S-adenosylhomocysteine are elevated in patients with

chronic kidney disease, which suggests a disturbance in the transsulfuration pathway or in the remethylation pathway. *Van Guldener et al.* observed that the homocysteine clearance by transsulfuration in patients with ESRD is severely decreased. However it is unclear whether the reduced homocysteine clearance by transsulfuration is the primary defect in renal failure or an adaptive response to the elevated homocysteine concentrations.[118]
Alternatively, it is hypothesized that an unidentified uraemic substance can inhibit the normal extrarenal homocysteine metabolism. This hypothesis is based on the assumption that the target organ is the liver because of its major regulatory role in the protein metabolism, its high levels of homocysteine metabolizing enzymes and its capacity to export high amounts of homocysteine *in-vitro*.[119]

Homocysteine and risk for cardiovascular disease and mortality in patients with CKD

Although homocysteine has been identified as a risk factor for cardiovascular disease in the general population, studies in patients with renal failure are controversial.[60,120]
Several case-control studies, which were mainly carried out in the nineties, reported higher homocysteine concentrations in patients with cardiovascular disease.[121-124] These results were confirmed by many prospective studies, which found a positive association between homocysteine levels and risk for cardiovascular disease and mortality.[112,125-128] However, in later studies on this topic an inverse relationship between homocysteine and cardiovascular disease as well as mortality was observed.[129,130] Besides differences in the study design and adjustment for confounders, the factors malnutrition and inflammation may explain the observed controversy. Several studies demonstrated that malnourished dialysis patients have lower homocysteine levels than patients with normal nutrition status.[130] It is suggested that homocysteine concentration is dependent on nutritional and inflammatory status. Therefore, in patients without malnutrition and inflammation homocysteine may be seen as a cardiovascular and mortality risk factor, whereas in malnourished and inflamed patients homocysteine may be a marker for better nutrition status and is associated with better survival.[131]

B vitamins in patients with CKD

Lower intake due to dietary restrictions, diminished appetite and increased losses due to the dialysis process account for deficiencies in water-soluble vitamins in patients with ESRD

treated with dialysis. Most water-soluble vitamins are molecules with molecular weight ranging from small to medium and may be removed during dialysis.[132,133] Furthermore, due to the alterations in the metabolism as a consequence of uraemia, patients with renal failure tend to have higher vitamin requirements. Therefore, supplementation with water-soluble vitamins is partly carried out on a routine basis in ESRD patients. However, large regional variations in the percentage of patients obtaining water-soluble vitamins were observed. In the United States about two-thirds of the patients received vitamins, compared to 4% of the patients in the United Kingdom and 6% of the patients in Japan.[134] Only in 2007, recommendations on vitamin use in ESRD were published.[135]

A reduction of homocysteine concentrations due to B-vitamin supplements was also observed in patients with ESRD. After supplementation with folic acid in dosages between 1mg and 60mg per day a decrease of homocysteine level of about 30% was observed.[136,137] No significant difference in the homocysteine lowering effect was observed concerning the application form of folic acid (oral or intravenous).[138] Vitamin B12, when administered 1mg parenteral in a 4-week interval, is efficient to decrease homocysteine level by 10%.[139] A combined oral application with folic acid, vitamin B12 and vitamin B6 can reduce homocysteine levels in dialysis patients by 30 to 50%.[140-142] Despite this strong reduction, normalization of homocysteine in patients with ESRD seems to be very rare.[143] Furthermore, it was observed that there is a dose-dependent effect when folic acid dosages below 1 mg per day are used. Multivitamin preparations with very low amounts of folic acid (160µg) did not lower homocysteine level significantly.[142]

Effects of B vitamins on total mortality and cardiovascular disease in patients with CKD

In patients with chronic kidney diseases only a limited number of RCTs is presently available, which tested the influence of B vitamins on risk for cardiovascular events and total mortality.

A first indication for an association between water-soluble vitamin use and total mortality came from the Dialysis Outcomes and Practice Patterns Study (DOPPS) I, which is a prospective observational study evaluating the use of water-soluble vitamins among dialysis patients in France, Germany, Italy, Japan, Spain, the United Kingdom, and the United States. In this study an association between the use of water-soluble vitamins and a significantly reduced mortality risk was observed (RR 0.84; 95% CI, 0.76-0.94; p=0.001).[134]

However, this observation was not confirmed by the HOST trial (Homocysteinemia in Kidney and End Stage Renal disease). Patients with chronic kidney disease and end-stage renal

disease were included. The supplementation with folic acid, vitamin B12 and B6 did not improve survival in this RCT (HR 1.04; 95% CI, 0.91-1.18; p=0.6).[144] Additionally, the results of a *post-hoc* analysis of the HOPE-2 study (HOPE-2 renal), by which only patients with a GFR<60ml/min were analysed, indicated no reduction in mortality due to the B vitamins.[145]

The results of the intervention studies to investigate whether B vitamins may influence cardiovascular events are heterogeneous. The large RCTs, ASFAST (Atherosclerosis and Folic Acid Supplementation Trial), HOST and HOPE-2 renal, indicate no reduction of risk of cardiovascular morbidity and mortality in patients with renal failure.[144-146] Yet, in another study with a smaller number of patients and no placebo control, a reduced cardiovascular event rate was observed (p=0.05) in patients receiving folic acid supplements.[147]

So far, the effect of a supplementation with B vitamins on cardiovascular events and total mortality is unclear.

Scope of the thesis

The 'homocysteine hypothesis' indicates that already moderately elevated homocysteine blood concentrations are a risk factor for atherosclerotic disease. Several studies in the general population revealed a positive association between homocysteine and cardiovascular disease and confirmed the 'homocysteine hypothesis'. The B vitamin status is regarded as the primary determinant of the homocysteine level and consequently homocysteine levels can be lowered by supplementation with folic acid and vitamin B12.
Patients with ESRD suffer from a particular high risk for cardiovascular disease, which leads, amongst others, to the high mortality rate observed in these patients. Up to now, treatment of established risk factors, such as elevated LDL cholesterol level or hypertension did not improve survival significantly in patients with ESRD. Hence there is urgent need to evaluate new treatable risk factors. Practically all patients with ESRD have elevated homocysteine blood concentrations, which are up to 10fold higher than in the general population. An elevation at such extend is otherwise only found in patients with the metabolic disorder homocystinuria and not observed for other established risk factors, e.g. LDL cholesterol level which is similar to the concentrations in the general population.

1. State-of-the-art: Homocysteine as a risk factor in patients with ESRD

Although many studies examined the possible association between homocysteine and risk for cardiovascular disease and total mortality in patients with ESRD; the results are neither concordant nor conclusive. Therefore, there was a need for a systematic evaluation of the results of available studies. A meta-analysis of the data of the literature was conducted to answer the question whether homocysteine is related to the risk for total mortality and cardiovascular disease in dialysis patients or not (Chapter 2).

2. Vitamin losses during haemodialysis

Furthermore, compared to the general population, dialysis patients are at higher risk for lower vitamin intake due to dietary restrictions, diminished appetite and metabolic changes. Additionally loss of water-soluble vitamins during the dialysis session may increase the risk for vitamin deficiencies. Therefore, the washout of the water-soluble B vitamins and of homocysteine during the haemodialysis session was investigated. Levels of serum and red

blood cell folate, plasma pyridoxal-5'-phosphate, serum cobalamin, blood thiamine, blood riboflavin, and plasma homocysteine before and after haemodialysis treatment were measured. In this study, the effect of different dialyser membranes on the washout of the B vitamins and homocysteine was investigated (Chapter 3).

3. Influence of B vitamin supplementation on cardiovascular events and survival in dialysis patients

In several studies it has been observed that supplementation with B vitamins may lower homocysteine levels in patients with ESRD. It might be hypothesized that a supplementation with B vitamins can reduce the cardiovascular risk and improve survival in ESRD patients due to the homocysteine lowering effect or other beneficial effects. Therefore, a prospective observational study and furthermore a randomized controlled trial was initiated to determine the impact of a therapy with B vitamins on total mortality and cardiovascular morbidity and mortality in patients treated with haemodialysis (Chapter 4 to 6).

References

1. Selhub J, Miller JW. The pathogenesis of homocysteinemia: interruption of the coordinate regulation by S-adenosylmethionine of the remethylation and transsulfuration of homocysteine. Am J Clin Nutr. 1992; 55:131-138.
2. Ueland PM, Refsum H, Stabler SP, Malinow MR, Andersson A, Allen RH. Total homocysteine in plasma or serum: methods and clinical applications. Clin Chem. 1993; 39:1764-1779.
3. Finkelstein JD. Methionine metabolism in mammals. J Nutr Biochem. 1990; 1:228-237.
4. Finkelstein JD. The metabolism of homocysteine: pathways and regulation. Eur J Pediatr. 1998; 157:S40-S44.
5. Gregory 3rd JF: Bioavailability of folate. Eur J Clin Nutr. 1997; 51:S54-S59.
6. Brzezińska A, Wińska P, Balińska M. Cellular aspects of folate and antifolate membrane transport. Acta Biochim Pol. 2000; 47:735-749.
7. Bässler KH, Golly I, Loew D, Pietrzik K: Vitamin-Lexikon für Ärzte, Apotheker und Ernährungswissenschaftler. 3. Auflage. München/Jena. Verlag Urban & Fischer 2002.
8. Sauberlich HE, Kretsch MJ, Skala JH, Johnson HL, Taylor PC. Folate requirement and metabolism in nonpregnant women. Am J Clin Nutr. 1987; 46:1016-1028.
9. Mulinaire J, Cordero J, Erickson J, Berry R: Periconceptional use of multivitamins and the occurrence of neural tube defects. JAMA. 1988; 260: 3141-3145.

10. Rampersaud GC, Kauwell GP, Bailey LB. Folate: a key to optimizing health and reducing disease risk in the elderly. J Am Coll Nutr. 2003; 22:1-8.
11. DGE (Deutsche Gesellschaft für Ernährung), Österreichische Gesellschaft für Ernährung, Schweizerische Gesellschaft für Ernährung, Schweizerische Gesellschaft für Ernährungsforschung, Schweizerische Vereinigung für Ernährung (eds.): Referenzwerte für die Nährstoffzufuhr. Frankfurt/Main: Umschau Braus Verlag 2000.
12. Andres E, Loukili NH, Noel E et al. Vitamin B12 (cobalamin) deficiency in elderly patients. CMAJ. 2004; 171:251-259.
13. Matthews JH. Cobalamin and folate deficiency in the elderly. Baillères Clin Haematol. 1995; 54:245-253.
14. Dong MH, McGown EL, Schwenneker BW, Sauberlich HE. Thiamin, riboflavin, and vitamin B6 contents of selected foods as served. J Am Diet Assoc. 1980; 76:156-160.
15. Malinow MR, Bostom AG, Krauss RM. Homocyst(e)ine, diet, and cardiovascular diseases: a statement for healthcare professionals from the Nutrition Committee, American Heart Association. Circulation. 1999; 99:178-182.
16. de Bree A, Verschuren WM, Blom HJ, Kromhout D. Association between B vitamin intake and plasma homocysteine concentration in the general Dutch population aged 20-65 y. Am J Clin Nutr. 2001; 73:1027-1033.
17. de Bree A, Verschuren WM, Blom HJ, de Graaf-Hess A, Trijbels FJ, Kromhout D. The homocysteine distribution: (mis)judging the burden. J Clin Epidemiol. 2001; 54:462-469.
18. Refsum H, Nygård O, Kvåle G, Ueland PM, Vollset SE. The Hordaland homocysteine study: the opposite tails odds ratios reveal differential effects of gender and intake of vitamin supplements at high and low plasma total homocysteine concentrations. J Nutr. 1996; 126:S1244-S1248.
19. Selhub J, Jacques PF, Wilson PW, Rush D, Rosenberg IH. Vitamin status and intake as primary determinants of homocysteinemia in an elderly population. JAMA. 1993; 270:2693-2698.
20. Nygård O, Vollset SE, Refsum H et al. Total plasma homocysteine and cardiovascular risk profile. The Hordaland Homocysteine Study. JAMA. 1995; 274:1526-1533.
21. Dierkes J, Jeckel A, Ambrosch A, Westphal S, Luley C, Boeing H. Factors explaining the difference of total homocysteine between men and women in the European Investigation Into Cancer and Nutrition Potsdam study. Metabolism. 2001; 50:640-645.
22. Giltay EJ, Hoogeveen EK, Elbers JM, Gooren LJ, Asscheman H, Stehouwer CD. Effects of sex steroids on plasma total homocysteine levels: a study in transsexual males and females. J Clin Endocrinol Metab. 1998; 83:550-553.
23. Schneede J, Refsum H, Ueland PM. Biological and environmental determinants of plasma homocysteine. Semin Thromb Hemost. 2000; 26:263-279.
24. Rasmussen LB, Ovesen L, Bülow I, Knudsen N, Laurberg P, Perrild H. Folate intake, lifestyle factors, and homocysteine concentrations in younger and older women. Am J Clin Nutr. 2000; 72:1156-1163.
25. Jacques PF, Bostom AG, Wilson PW, Rich S, Rosenberg IH, Selhub J. Determinants of plasma total homocysteine concentration in the Framingham Offspring cohort. Am J Clin Nutr. 2001; 73:613-621.
26. Dallongeville J, Marécaux N, Fruchart JC, Amouyel P. Cigarette smoking is associated with unhealthy patterns of nutrient intake: a meta-analysis. J Nutr. 1998; 128:1450-1457.
27. Halsted CH. Lifestyle effects on homocysteine and an alcohol paradox. Am J Clin Nutr. 2001; 73:501-502.

28. Cravo ML, Glória LM, Selhub J et al. Hyperhomocysteinemia in chronic alcoholism: correlation with folate, vitamin B-12, and vitamin B-6 status. Am J Clin Nutr. 1996; 63:220-224.
29. Baker H, Leevy CB, DeAngelis B, Frank O, Baker ER. Cobalamin (vitamin B12) and holotranscobalamin changes in plasma and liver tissue in alcoholics with liver disease. J Am Coll Nutr. 1998; 17:235-238.
30. Gudnason V, Stansbie D, Scott J, Bowron A, Nicaud V, Humphries S. C677T (thermolabile alanine/valine) polymorphism in methylenetetrahydrofolate reductase (MTHFR): its frequency and impact on plasma homocysteine concentration in different European populations. EARS group. Atherosclerosis. 1998; 136:347-354.
31. Christensen B, Mosdol A, Retterstol L, Landaas S, Thelle DS. Abstention from filtered coffee reduces the concentrations of plasma homocysteine and serum cholesterol-a randomized controlled trial. Am J Clin Nutr. 2001; 74:302-307.
32. Grubben MJ, Boers GH, Blom HJ et al. Unfiltered coffee increases plasma homocysteine concentrations in healthy volunteers: a randomized trial. Am J Clin Nutr. 2000; 71:480-484.
33. Olthof MR, Hollman PC, Zock PL, Katan MB. Consumption of high doses of chlorogenic acid, present in coffee, or of black tea increases plasma total homocysteine concentrations in humans. Am J Clin Nutr. 2001; 73:532-538.
34. Nurk E, Tell GS, Nygård O, Refsum H, Ueland PM, Vollset SE. Plasma total homocysteine is influenced by prandial status in humans: the Hordaland Homocysteine Study. J Nutr. 2001; 131:1214-1216.
35. Lambie DG, Johnson RH. Drugs and folate metabolism. Drugs. 1985; 30:145-155.
36. Refsum H, Ueland PM. Clinical significance of pharmacological modulation of homocysteine metabolism. Trends Pharmacol Sci. 1990; 11:411-416.
37. Dierkes J, Westphal S, Luley C. Serum homocysteine increases after therapy with fenofibrate or bezafibrate. Lancet. 1999; 354:219-220.
38. Dierkes J, Westphal S. Effect of drugs on homocysteine concentrations. Semin Vasc Med. 2005; 5:124-139.
39. Frosst P, Blom HJ, Milos R et al. A candidate genetic risk factor for vascular disease: a common mutation in methylenetetrahydrofolate reductase. Nat Genet. 1995; 10:111-113.
40. Brattström L, Zhang Y, Hurtig M et al. A common methylenetetrahydrofolate reductase gene mutation and longevity. Atherosclerosis. 1998; 141:315-319.
41. Guenther BD, Sheppard CA, Tran P, Rozen R, Matthews RG, Ludwig ML. The structure and properties of methylenetetrahydrofolate reductase from Escherichia coli suggest how folate ameliorates human hyperhomocysteinemia. Nat Struct Biol. 1999; 6:359-365.
42. Geisel J, Zimbelmann I, Schorr H, et al. Genetic defects as important factors for moderate hyperhomocysteinemia. Clin Chem Lab Med. 2001; 39:698-704.
43. Lievers KJ, Kluijtmans LA, Blom HJ. Genetics of hyperhomocysteinaemia in cardiovascular disease. Ann Clin Biochem. 2003; 40:46-59.
44. Heil SG, Van der Put NM, Waas ET, den Heijer M, Trijbels FJ, Blom HJ. Is mutated serine hydroxymethyltransferase (SHMT) involved in the etiology of neural tube defects? Mol Genet Metab. 2001; 73:164-172
45. Namour F, Olivier J, Abdelmouttaleb I et al. Transcobalamin codon 259 polymorphism in HT-29 and Caco-2 cells and in Caucasians: relation to transcobalamin and homocysteine concentration in blood. Blood. 2001; 97:1092-1098.

46. Wans S, Schüttler K, Jakubiczka S, Müller A, Luley C, Dierkes J. Analysis of the transcobalamin II 776C>G (259P>R) single nucleotide polymorphism by denaturing HPLC in healthy elderly: associations with cobalamin, homocysteine and holo-transcobalamin II. Clin Chem Lab Med. 2003; 41:1532-1536.
47. Kawakami K, Omura K, Kanehira E, Watanabe Y. Polymorphic tandem repeats in the thymidylate synthase gene is associated with its protein expression in human gastrointestinal cancers. Anticancer Res. 1999; 19:3249-3252.
48. DeMille MM, Kidd JR, Ruggeri V et al. Population variation in linkage disequilibrium across the COMT gene considering promoter region and coding region variation. Hum Genet. 2002; 111:521-537.
49. Devlin AM, Ling EH, Peerson JM et al. Glutamate carboxypeptidase II: a polymorphism associated with lower levels of serum folate and hyperhomocysteinemia. Hum Mol Genet. 2000; 9:2837-2844.
50. Chango A, Emery-Fillon N, de Courcy GP et al. A polymorphism (80G->A) in the reduced folate carrier gene and its associations with folate status and homocysteinemia. Mol Genet Metab. 2000; 70:310-315.
51. Mudd SH, Levy HL, Skovby F. Disorders of transsulfuration. In: Scriver CR, Beaudet AL, Sly WS, Valle D, eds. The Metabolic and Molecular Bases of Inherited Disease. 7th ed. New York, NY: McGraw-Hill; 1995: 1279–1327.
52. Naughten ER, Yap S, Mayne PD. Newborn screening for homocystinuria: Irish and worldwide experience. Eur J Pediatr. 1998; 157:S84–S87.
53. Mudd SH, Finkelstein JD, Irreverre F, Laster L. Homocysteinuria: An enzymatic Defect. Science. 1964; 143:1443-1445.
54. Mudd SH, Skovby F, Levy HL et al. The natural history of homocystinuria due to cystathionine beta-synthase deficiency. Am J Hum Genet. 1985; 37:1-31.
55. McCully KS. Homocysteine and vascular disease. Nat Med. 1996; 2:386-389.
56. Yap S, Boers GH, Wilcken B et al. Vascular outcome in patients with homocystinuria due to cystathionine beta-synthase deficiency treated chronically: a multicenter observational study. Arterioscler Thromb Vasc Biol. 2001; 21:2080-2085.
57. McCully KS. Vascular pathology of homocysteinemia: implications for the pathogenesis of arteriosclerosis. Am J Pathol. 1969; 56:111-128.
58. Boushey CJ, Beresford SA, Omenn GS, Motulsky AG. A quantitative assessment of plasma homocysteine as a risk factor for vascular disease. Probable benefits of increasing folic acid intakes. JAMA. 1995; 274:1049-1057.
59. Danesh J, Lewington S. Plasma homocysteine and coronary heart disease: systematic review of published epidemiological studies. J Cardiovasc Risk. 1998; 5:229-232.
60. Homocysteine Studies Collaboration. Homocysteine and risk of ischemic heart disease and stroke: a meta-analysis. JAMA. 2002; 288:2015-2022.
61. Casas JP, Bautista LE, Smeeth L, Sharma P, Hingorani AD. Homocysteine and stroke: evidence on a causal link from mendelian randomisation. Lancet. 2005; 365:224-232.
62. Van den Berg M, Boers GH, Franken DG et al. Hyperhomocysteinaemia and endothelial dysfunction in young patients with peripheral arterial occlusive disease. Eur J Clin Invest. 1995; 25:176-181.
63. Perna AF, Ingrosso D, De Santo NG. Homocysteine and oxidative stress. Amino Acids. 2003; 25:409-417.
64. Loscalzo J. The oxidant stress of hyperhomocyst(e)inemia. J Clin Invest. 1996; 98:5-7.

65. Lynch SM, Campione AL, Moore MK. Plasma thiols inhibit hemin-dependent oxidation of human low-density lipoprotein. Biochim Biophys Acta. 2000; 1485:11-22.
66. Wilcken DE, Wang XL, Adachi T et al. Relationship between homocysteine and superoxide dismutase in homocystinuria: possible relevance to cardiovascular risk. Arterioscler Thromb Vasc Biol. 2000; 20:1199-1202.
67. Perna AF, Ingrosso D, Lombardi C et al. Possible mechanisms of homocysteine toxicity. Kidney Int Suppl. 2003; 84:S137-S140.
68. Upchurch GR Jr, Welch GN, Fabian AJ et al. Homocyst(e)ine decreases bioavailable nitric oxide by a mechanism involving glutathione peroxidase. J Biol Chem. 1997; 272:17012-17017.
69. Jakubowski H. The synthetic/editing active site of an aminoacyl-tRNA synthetase: evidence for binding of thiols in the editing subsite. Biochemistry. 1996; 35:8252-8259.
70. Dong C, Yoon W, Goldschmidt-Clermont PJ. DNA methylation and atherosclerosis. J Nutr. 2002; 132:S2406-S2409.
71. Bestor TH. The DNA methyltransferases of mammals. Hum Mol Genet. 2000; 9:2395-2402.
72. Vilkaitis G, Merkiene E, Serva S, Weinhold E, Klimasauskas S. The mechanism of DNA cytosine-5 methylation. Kinetic and mutational dissection of Hhai methyltransferase. J Biol Chem. 2001 15; 276:20924-20934.
73. Castro R, Rivera I, Martins C et al. Intracellular S-adenosylhomocysteine increased levels are associated with DNA hypomethylation in HUVEC. J Mol Med. 2005; 83:831-836.
74. Lubec B, Labudova O, Hoeger H et al. Homocysteine increases cyclin-dependent kinase in aortic rat tissue. Circulation. 1996; 94:2620-2625.
75. Chambers JC, Obeid OA, Kooner JS. Physiological increments in plasma homocysteine induce vascular endothelial dysfunction in normal human subjects. Arterioscler Thromb Vasc Biol. 1999; 19:2922-2927.
76. Chambers JC, McGregor A, Jean-Marie J, Kooner JS. Acute hyperhomocysteinaemia and endothelial dysfunction. Lancet. 1998; 351:36-37.
77. Stamler JS, Osborne JA, Jaraki O et al. Adverse vascular effects of homocysteine are modulated by endothelium-derived relaxing factor and related oxides of nitrogen. J Clin Invest. 1993; 91:308-318.
78. Demuth K, Atger V, Borderie D et al. Homocysteine decreases endothelin-1 production by cultured human endothelial cells. Eur J Biochem. 1999; 263:367-376.
79. Homocysteine Lowering Trialists' Collaboration. Dose-dependent effects of folic acid on blood concentrations of homocysteine: a meta-analysis of the randomized trials. Am J Clin Nutr. 2005; 82:806-812.
80. Schnyder G, Roffi M, Pin R et al. Decreased rate of coronary restenosis after lowering of plasma homocysteine levels. N Engl J Med. 2001; 345:1593-1600.
81. Schnyder G, Roffi M, Flammer Y, Pin R, Hess OM. Effect of homocysteine-lowering therapy with folic acid, vitamin B12, and vitamin B6 on clinical outcome after percutaneous coronary intervention: the Swiss Heart study: a randomized controlled trial. JAMA. 2002; 288:973-979.
82. Baker F, Picton D, Blackwood S, et al. Blinded comparison of folic acid and placebo in patients with ischemic heart disease: an outcome trial. Circulation 2002; 106:3642 (abstr).

83. Toole JF, Malinow MR, Chambless LE et al. Lowering homocysteine in patients with ischemic stroke to prevent recurrent stroke, myocardial infarction, and death. The Vitamin Intervention for Stroke Prevention (VISP) randomized controlled trial. JAMA. 2004; 291:565-575.
84. Bønaa KH, Njølstad I, Ueland PM et al. Homocysteine lowering and cardiovascular events after acute myocardial infarction. N Engl J Med. 2006; 354:1578-1588.
85. Ebbing M, Bleie Ø, Ueland PM et al. Mortality and cardiovascular events in patients treated with homocysteine-lowering B vitamins after coronary angiography: a randomized controlled trial. JAMA. 2008; 300:795-804.
86. Lonn E, Yusuf S, Arnold MJ et al. Homocysteine lowering with folic acid and B vitamins in vascular disease. N Engl J Med. 2006; 354:1567-1577.
87. Albert CM, Cook NR, Gaziano JM et al. Effect of folic acid and B vitamins on risk of cardiovascular events and total mortality among women at high risk for cardiovascular disease: a randomized trial. JAMA. 2008; 299:2027-2036.
88. Armitage JM, SEARCH Study Collaborative Group. SEARCH (Study of the Effectiveness of Additional Reductions in Cholesterol and Homocysteine): Randomized Comparison of Folic Acid 2 mg Plus Vitamin B12 1 mg Daily versus Placebo for 7 Years in 12,064 Myocardial Infarction Survivors. Circulation. 2008; 118:2310 (abstr)
89. Bazzano LA, Reynolds K, Holder KN, He J. Effect of folic acid supplementation on risk of cardiovascular diseases: a meta-analysis of randomized controlled trials. JAMA. 2006; 296:2720-2726.
90. Wang X, Qin X, Demirtas H et al. Efficacy of folic acid supplementation in stroke prevention: a meta-analysis. Lancet. 2007; 369: 1876-1882.
91. Smith HW. Diseases of the kidney and urinary tract. In: The Kidney: Structure and Function in Health and Disease. New York: Oxford Univ Pr; 1951:836-887.
92. Cockcroft DW, Gault MH: Prediction of creatinine clearance from serum creatinine. Nephron. 1976; 16:31-41.
93. Levey AS, Bosch JP, Lewis JB, Greene T, Rogers N, Roth D. A more accurate method to estimate glomerular filtration rate from serum creatinine: a new prediction equation. Modification of Diet in Renal Disease Study Group. Ann Intern Med. 1999; 130:461-470.
94. Shaymann JA. Renal Pathophysiology. 1995; J.B. Lippincott Company, Philadelphia.
95. National Kidney Foundation. K/DOQI clinical practice guidelines for chronic kidney disease: evaluation, classification, and stratification. Am J Kidney Dis. 2002; 39:S1-S266.
96. Frei U, Schober-Halstenberg HJ. Nierenersatztherapie in Deutschland: Bericht über Dialysebehandlung und Nierentransplantation in Deutschland. 2006/2007
97. Foley RN, Parfrey PS, Sarnak MJ. Epidemiology of cardiovascular disease in chronic renal disease. J Am Soc Nephrol. 1998; 9: S16-S23,
98. Sarnak MJ, Levey AS, Schoolwerth AC et al. Kidney disease as a risk factor for development of cardiovascular disease: a statement from the American Heart Association Councils on Kidney in Cardiovascular Disease, High Blood Pressure Research, Clinical Cardiology, and Epidemiology and Prevention. Circulation. 2003; 108:2154-2169.
99. Wilson PW, D'Agostino RB, Levy D, Belanger AM, Silbershatz H, Kannel WB. Prediction of coronary heart disease using risk factor categories. Circulation. 1998; 97:1837-1847.

100. Menon V, Sarnak MJ. The epidemiology of chronic kidney disease stages 1 to 4 and cardiovascular disease: a high-risk combination. Am J Kidney Dis. 2005; 45:223-232.
101. Orth SR, Ritz E, Schrier RW. The renal risks of smoking. Kidney Int. 1997; 51:1669-1677.
102. Prichard S. Risk factors for coronary artery disease in patients with renal failure. Am J Med Sci. 2003; 325:209-213.
103. Foley RN, Parfrey PS, Harnett JD, Kent GM, Murray DC, Barre PE. Impact of hypertension on cardiomyopathy, morbidity and mortality in end-stage renal disease. Kidney Int. 1996; 49:1379-1385.
104. Klassen PS, Lowrie EG, Reddan DN et al. Association between pulse pressure and mortality in patients undergoing maintenance hemodialysis. JAMA. 2002; 287:1548-1555.
105. Kalantar-Zadeh K, Kilpatrick RD, McAllister CJ, Greenland S, Kopple JD. Reverse epidemiology of hypertension and cardiovascular death in the hemodialysis population: the 58th annual fall conference and scientific sessions. Hypertension. 2005; 45:811-817.
106. Nishizawa Y, Shoji T, Ishimura E, Inaba M, Morii H. Paradox of risk factors for cardiovascular mortality in uremia: is a higher cholesterol level better for atherosclerosis in uremia? Am J Kidney Dis. 2001; 38:S4-S7.
107. Salahudeen AK. So, overweight is associated with better survival in dialysis patients! Nephrol Dial Transplant. 2002; 17:1151-1152.
108. Kalantar-Zadeh K. What is so bad about reverse epidemiology anyway? Semin Dial. 2007; 20:593-601.
109. Kalantar-Zadeh K, Ikizler TA, Block G, Avram MM, Kopple JD. Malnutrition-inflammation complex syndrome in dialysis patients: causes and consequences. Am J Kidney Dis. 2003; 42:864-881.
110. Menon V, Gul A, Sarnak MJ. Cardiovascular risk factors in chronic kidney disease. Kidney Int. 2005; 68:1413-1418.
111. Knight EL, Rimm EB, Pai JK et al. Kidney dysfunction, inflammation, and coronary events: a prospective study. J Am Soc Nephrol. 2004; 15:1897-1903.
112. Dierkes J, Domröse U, Westphal S et al. Cardiac troponin T predicts mortality in patients with end-stage renal disease. Circulation. 2000; 102:1964-1969.
113. Kielstein JT, Salpeter SR, Buckley NS, Cooke JP, Fliser D. Two cardiovascular risk factors in one? Homocysteine and its relation to glomerular filtration rate. A meta-analysis of 41 studies with 27,000 participants. Kidney Blood Press Res. 2008; 31:259-267.
114. Refsum H, Helland S, Ueland PM. Radioenzymic determination of homocysteine in plasma and urine. Clin Chem. 1985; 31:624-628.
115. van Guldener C, Donker AJ, Jakobs C, Teerlink T, de Meer K, Stehouwer CD. No net renal extraction of homocysteine in fasting humans. Kidney Int. 1998; 54:166-169.
116. Ghandour H, Bagley PJ, Shemin D et al. Distribution of plasma folate forms in hemodialysis patients receiving high daily doses of L-folinic or folic acid. Kidney Int. 2002; 62:2246-2249.
117. Födinger M, Mannhalter C, Wölfl G et al. Mutation (677 C to T) in the methylenetetrahydrofolate reductase gene aggravates hyperhomocysteinemia in hemodialysis patients. Kidney Int. 1997; 52:517-523.
118. van Guldener C, Kulik W, Berger R et al. Homocysteine and methionine metabolism in ESRD: A stable isotope study. Kidney Int. 1999; 56:1064-1071.
119. van Guldener C. Why is homocysteine elevated in renal failure and what can be expected from homocysteine-lowering? Nephrol Dial Transplant. 2006; 21:1161-1166.

120. Wald DS, Law M, Morris JK. Homocysteine and cardiovascular disease: evidence on causality from a meta-analysis. BMJ. 2002; 325:1202-1206.
121. Bachmann J, Tepel M, Raidt H et al. Hyperhomocysteinemia and the risk for vascular disease in hemodialysis patients. J Am Soc Nephrol. 1995; 6:121-125.
122. Robinson K, Gupta A, Dennis V et al. Hyperhomocysteinemia confers an independent increased risk of atherosclerosis in end-stage renal disease and is closely linked to plasma folate and pyridoxine concentrations. Circulation. 1996; 94:2743-2748.
123. Kunz K, Petitjean P, Lisri M et al. Cardiovascular morbidity and endothelial dysfunction in chronic haemodialysis patients: is homocyst(e)ine the missing link? Nephrol Dial Transplant. 1999; 14:1934-1942.
124. Manns BJ, Burgess ED, Hyndman ME, Parsons HG, Schaefer JP, Scott-Douglas NW. Hyperhomocyst(e)inemia and the prevalence of atherosclerotic vascular disease in patients with end-stage renal disease. Am J Kidney Dis. 1999; 34:669-677.
125. Mallamaci F, Zoccali C, Tripepi G et al. Hyperhomocysteinemia predicts cardiovascular outcomes in hemodialysis patients. Kidney Int. 2002; 61:609-614.
126. Moustapha A, Naso A, Nahlawi M et al. Prospective study of hyperhomocysteinemia as an adverse cardiovascular risk factor in end-stage renal disease. Circulation. 1998; 97:138-141.
127. Haraki T, Takegoshi T, Kitoh C et al. Hyperhomocysteinemia, diabetes mellitus, and carotid atherosclerosis independently increase atherosclerotic vascular disease outcome in Japanese patients with end-stage renal disease. Clin Nephrol. 2001; 56:132-139.
128. Ducloux D, Bresson-Vautrin C, Kribs M, Abdelfatah A, Chalopin JM. C-reactive protein and cardiovascular disease in peritoneal dialysis patients. Kidney Int. 2002; 62:1417-1422.
129. Kalantar-Zadeh K, Block G, Humphreys MH, McAllister CJ, Kopple JD. A low, rather than a high, total plasma homocysteine is an indicator of poor outcome in hemodialysis patients. J Am Soc Nephrol. 2004; 15:442-453.
130. Ducloux D, Klein A, Kazory A, Devillard N, Chalopin JM. Impact of malnutrition-inflammation on the association between homocysteine and mortality. Kidney Int. 2006; 69:331-335.
131. Suliman ME, Qureshi AR, Barany P et al. Hyperhomocysteinemia, nutritional status, and cardiovascular disease in hemodialysis patients. Kidney Int. 2000; 57:1727-1735.
132. Lasseur C, Parrot F, Delmas Y, et al. Impact of high-flux/high-efficiency dialysis on folate and homocysteine metabolism. J. Nephrol. 2001; 14:32-35.
133. De Vriese AS, Langlois M, Bernard D et al. Effect of dialyser membrane pore size on plasma homocysteine levels in haemodialysis patients. Nephrol. Dial. Transplant. 2003; 18:2596-2600.
134. Fissell RB, Bragg-Gresham JL, Gillespie BW et al. International variation in vitamin prescription and association with mortality in the Dialysis Outcomes and Practice Patterns Study (DOPPS). Am J Kidney Dis. 2004; 44:293-299.
135. Fouque D, Vennegoor M, ter Wee P, et al. EBPG guideline on nutrition. Nephrol. Dial. Transplant. 2007; 22:ii45-ii87.
136. Yango A, Shemin D, Hsu N et al. Rapid communication: L-folinic acid versus folic acid for the treatment of hyperhomocysteinemia in hemodialysis patients. Kidney Int. 2001; 59:324-327.

137. Koyama K, Usami T, Takeuchi O, Morozumi K, Kimura G. Efficacy of methylcobalamin on lowering total homocysteine plasma concentrations in haemodialysis patients receiving high-dose folic acid supplementation. Nephrol Dial Transplant. 2002; 17:916-922.
138. Ducloux D, Aboubakr A, Motte G et al. Hyperhomocysteinaemia therapy in haemodialysis patients: folinic versus folic acid in combination with vitamin B6 and B12. Nephrol Dial Transplant. 2002; 17:865-870.
139. Kaplan LN, Mamer OA, Hoffer LJ. Parenteral vitamin B12 reduces hyperhomocysteinemia in end-stage renal disease. Clin Invest Med 2001; 24: 5–11.
140. Henning BF, Zidek W, Riezler R, Graefe U, Tepel M. Homocyst(e)ine metabolism in hemodialysis patients treated with vitamins B6, B12 and folate. Res Exp Med. 2001; 200:155-168.
141. Suliman ME, Divino Filho JC, Bàràny P, Anderstam B, Lindholm B, Bergström J. Effects of high-dose folic acid and pyridoxine on plasma and erythrocyte sulfur amino acids in hemodialysis patients. J Am Soc Nephrol. 1999; 10:1287-1296.
142. Dierkes J, Domröse U, Bosselmann KP, Neumann KH, Luley C. Homocysteine lowering effect of different multivitamin preparations in patients with end-stage renal disease. J Ren Nutr. 2001; 11:67-72.
143. Bostom AG, Shemin D, Gohh RY et al. Treatment of mild hyperhomocysteinemia in renal transplant recipients versus hemodialysis patients. Transplantation. 2000; 69:2128-2131.
144. Jamison RL, Hartigan P, Kaufman JS et al. Effect of homocysteine lowering on mortality and vascular disease in advanced chronic kidney disease and end-stage renal disease: a randomized controlled trial. JAMA. 2007; 298:1163-1170.
145. Mann JF, Sheridan P, McQueen MJ et al. HOPE-2 investigators. Homocysteine lowering with folic acid and B vitamins in people with chronic kidney disease--results of the renal Hope-2 study. Nephrol Dial Transplant. 2008; 23:645-653.
146. Zoungas S, McGrath BP, Branley P et al. Cardiovascular morbidity and mortality in the Atherosclerosis and Folic Acid Supplementation Trial (ASFAST) in chronic renal failure: a multicenter, randomized, controlled trial. J Am Coll Cardiol. 2006; 47:1108-1116.
147. Righetti M, Serbelloni P, Milani S, Ferrario G. Homocysteine-lowering vitamin B treatment decreases cardiovascular events in hemodialysis patients. Blood Purif. 2006; 24: 379-386.

2

Homocysteine as a Risk Factor for Cardiovascular Disease in Patients Treated by Dialysis: A Meta-Analysis.

Based on the original article:

Heinz J, Kropf S, Luley C, Dierkes J. Homocysteine as a Risk Factor for Cardiovascular Disease in Patients Treated by Dialysis: A Meta-Analysis. Am J Kidney Dis. 2009; Apr 7 *Epub ahead of print*

For reasons of copyright, the original article is only included in abstract-form in the published version of this dissertation. The original article is available from the "American Journal of Kidney Diseases".

Abstract

Background:

In the general population elevated homocysteine concentrations are a risk factor for cardiovascular disease and mortality. However, it is not known whether this also applies to patients with end-stage renal disease.

Study Design:

Meta-analysis of retrospective (11 studies including 1506 individuals), prospective observational studies (12 studies including 1975 individuals) and intervention trials (5 studies including 1642 dialysis patients). Analyses were carried out separately, according to the study design.

Setting and Population:

Studies in patients with end-stage renal disease treated by haemodialysis or peritoneal dialysis.

Selection Criteria for Studies:

Studies investigating the association between total homocysteine and cardiovascular disease or total mortality, or the influence of a vitamin supplementation on the cardiovascular or mortality risk.

Intervention:

In the intervention studies vitamin preparations with folic acid alone or in combination with other vitamins, as vitamin B12 and B6 were used.

Outcomes:

In the retrospective studies 'cases' are patients with cardiovascular diseases. The outcomes for the prospective observational and intervention studies are cardiovascular events and total mortality.

Results:

In the retrospective studies, there was no significant overall difference in homocysteine concentrations between 'cases' and controls (weighted mean difference of homocysteine 2.82µmol/L; 95% CI, -2.22-7.86; p=0.3). The pooled overall risk estimate for the prospective observational studies suggests no association between homocysteine (5µmol/L increase) and total mortality (HR 1.02; 95% CI, 0.93-1.12; p=0.7), but there was an association with cardiovascular events (HR 1.09; 95% CI, 1.03-1.14; p=0.001). In a subgroup analysis of patients not receiving vitamins, an increase in homocysteine was associated increased mortality (HR 1.07; 95% CI, 1.02-1.13; p=0.01).

For the intervention trials with B-vitamins there was a significant risk reduction for the cardiovascular disease (RR 0.73; 95% CI, 0.56-0.94; p=0.02) but no risk reduction for total mortality or the composite endpoint including total mortality (RR 1.01; 95% CI, 0.88-1.15; p=0.9).

Limitations:

Many studies are small size which may lead to the observed heterogeneity. Some of the intervention trials are neither placebo-controlled nor randomized. Separate analyses for specific endpoints and patients treated with haemodialysis or peritoneal dialysis were not possible.

Conclusion:

Total homocysteine may be a risk factor for cardiovascular events and for total mortality in patients with end-stage renal disease not receiving vitamin supplementation or folic acid food fortification. There may be a potential for reducing cardiovascular disease in this population group by folic acid supplementation.

Key words: cardiovascular diseases, dialysis, homocysteine, mortality, risk factors

Acknowledgements

We are indebted to all participating authors for providing patients' data for the present meta-analysis:

Killian Robinson, Robert Clarke, Anjan Gupta, Vincent W. Dennis (Cleveland, USA; Oxford, England)
Gérard M. London, Jacques Blacher (Fleury-Mérogis, France)
Ramón Romero, Beatriu Bayés (Badalona, Spain)
Braden J. Manns, Nairne Scott Douglas (Calgary, Canada)
Sandra Sirrs, Adeera Levin, Ognjenka Djurdjev (Vancouver, Canada)
Francesca Mallamaci, Carmine Zoccali, Giovanni Tripepi (Reggio Calabria, Italy)
Gere Sunder-Plassmann, Andreas Vychytil (Vienna, Austria)

and to Dirk Hasenclever from the Institute for Medical Informatics, Statistics and Epidemiology (IMISE) of the University Leipzig, Germany for the encouragement of the idea of this project and for discussion of the design of this meta-analysis.

3

Washout of Water-Soluble Vitamins and of Homocysteine during Haemodialysis – Effect of High-Flux and Low-Flux Dialyser Membranes

Based on the original article:

Heinz J, Domröse U, Westphal S, Luley C, Neumann KH, Dierkes J. Washout of water-soluble vitamins and of homocysteine during haemodialysis: effect of high-flux and low-flux dialyser membranes. Nephrology. 2008; 13:384-389.

Abstract

Background:

Vitamin deficiencies are common in patients with end-stage renal disease owing to dietary restrictions, drug-nutrient interactions, changes in metabolism, and vitamin losses during dialysis. The present study investigated the levels of serum and red blood cell (RBC) folate, plasma pyridoxal-5'-phosphate (PLP), serum cobalamin, blood thiamine, blood riboflavin, and plasma homocysteine (tHcy) before and after haemodialysis treatment.

Methods:

Vitamin and tHcy blood concentrations were measured in 30 patients with ESRD before and after dialysis session either with low-flux (n=15) or high-flux (n=15) dialysers.

Results:

After the dialysis procedure significant lower concentrations of serum folate (37%), plasma PLP (35%), blood thiamine (6%), and blood riboflavin (7%) were observed. No significant changes were found for serum cobalamin or for RBC folate. There were no differences in the washout of water-soluble vitamins between treatments with low-flux and high-flux membranes. Furthermore, a 41% lower concentration in tHcy was observed. The percentage decrease in tHcy was significantly greater in the patients treated with high-flux dialysers (48% vs. 37%; $p<0.01$). The percentage change during dialysis was significantly inversely related to the molecular weight of the vitamins measured ($r=-0.867$, $p<0.01$).

Conclusions:

This study showed significantly lower blood or serum levels of various water-soluble vitamins after dialysis, independently of the dialyser membrane. The monitoring of the vitamin status is essential in patients treated with high-flux dialysers as well as in patients treated with low-flux dialysers.

Keywords: haemodialysis, high-flux, homocysteine, low-flux, water-soluble vitamins

Abbreviations:

ESRD	End-stage renal disease
tHcy	Total homocysteine (sum of free and protein-bound homocysteine)
EDTA	Ethylenediaminetetraacetic acid
PA	Polyamide
PAES	Polyarylethersulfone
PVP	Polyvinylpyrrolidone
PES	Polyethersulfone
HPLC	High-performance liquid chromatography
RBC	Red blood cell
FAD	Flavin adenine dinucleotide
FMN	Flavin mononucleotide
PLP	Pyridoxal-5'-phosphate
LF	Low-flux dialysis
HF	High-flux dialysis
Kt/V_{urea}	Urea nitrogen clearance divided by volume of distribution of urea nitrogen

Introduction

End-stage renal disease (ESRD) is associated with an age-adjusted mortality rate 3.5-4 times that in the general population, mainly because of increased cardiovascular mortality.[1,2] Among other factors, the nutritional status is believed to play a key role in determining survival.[3-5] The components of nutritional status in ESRD patients that may give cause for concern are the protein and amino acid metabolism and the vitamin status.

Vitamin supplementation with water-soluble vitamins is widely used in ESRD patients to counteract the restricted intake, increased losses, and altered metabolism. As the percentage of patients receiving supplements of water-soluble vitamins varies widely,[6] first guidelines on vitamin use in ESRD were published in 2007.[7] Although observational studies have shown that vitamin supplementation may reduce mortality in ESRD patients,[6,8] the results of the first randomized controlled trial with B-vitamins intervention in patients with advanced chronic kidney disease and ESRD did not confirm this observation.[9]

In many dialysis centres the use of standard dialysers has been changed to high-flux dialysers. According to the HEMO study, 60% of all patients are treated with high-flux dialysers.[10] High-flux membranes have a number of advantages, such as better clearances of larger molecules. However many vitamins fall into a category between small and middle molecular weights and may be removed by high-flux dialysis. Losses of water-soluble vitamins during dialysis treatment especially with high-flux membranes over a period of 1-3 months have been investigated in recent studies, most of these focusing on folate and vitamin B6.[11-14] The interest in these vitamins is due to their involvement into the metabolism of homocysteine, an atherogenic sulphur-containing amino acid which is found to be strongly elevated in ESRD.[15] It has already been shown that homocysteine can be removed by dialysis, but there is a lack of studies concerned with both homocysteine and related vitamins and with their changes during dialysis as a function of dialyser membrane type.

We therefore decided to investigate the changes in the blood concentrations of the B-vitamins and of homocysteine during the dialysis procedure, and also the difference in vitamin losses between high-flux and low-flux membranes.

Patients and methods

Thirty haemodialysis patients (11 females, 19 males) were selected from the Outpatients Dialysis Centre in Magdeburg, where they were haemodialysed 3 times a week (Table 1).
The median time of dialysis sessions was 12.5 (9.0-16.5) and differed not between high-flux and low-flux dialysis. The sessions started at different times of the day (in the morning, in the midday and in the high-flux group also in the evening). During dialysis patients were allowed to eat a non-standardized meal, which usually consisted of a bread roll with margarine and cold meat.
Blood flow was 280 or 300mL/min in both groups. Dialysates used were D 263 (Gambro; composition: potassium 4.0mmol/L; calcium 1.25mmol/L; chloride 110.5mmol/L; magnesium 0.5mmol/L; glucose 1.0g/L; sodium 138.0mmol/L; bicarbonate 32.0mmol/L and acetate 3.0mmol/L) or D 283 (Gambro; composition: potassium 3.0mmol/L; calcium 1.25mmol/L; chloride 109.5mmol/L; magnesium 0.5mmol/L; glucose 1.0g/L; sodium 138.0mmol/L; bicarbonate 32.0mmol/L and acetate 3.0mmol/L).

Table 1: Dialyser characteristics

	Low-flux dialyser †	High-flux dialyser ‡
Effective surface area (range; m²)	1.3 - 1.4	1.5 - 1.8
UF coefficient (range; mL/h/mmHg) §	10 - 13	43 - 60
Blood flow (range; mL/min)	150 - 400	200 - 400
Dialysate flow (range; mL/min)	500 - 800	500 - 800
Clearance (range; mL/min)		
Urea	186 - 319	192 - 351
Creatinine	171 - 264	180 - 311
Phosphate	152 - 221	174 - 295
Vitamin B12	90 - 114	128 - 204

† Polysulfone membrane F6 HPS (Fresenius Medical Care AG, Germany); Polyamix (PAES/PVP/PA) membrane Polyflux 14L (Gambro, Germany)

‡ Polysulfone membrane F80 S (Fresenius Medical Care AG); Helixone (Polysulfone/PVP blend) membrane FX60 (Fresenius Medical Care AG); Polyamix (PAES/PVP/PA) membrane Polyflux 140H (Gambro); Diapes (PES) membrane PES-150DH (Nipro, Japan)

§ UF = ultrafiltration

Fluid removal during dialysis session was calculated by the weight difference before and after dialysis and was significantly different between the high and low flux group. The median (5^{th}-

95th perc.) fluid removal in the high flux group was 3.78kg (0.39-5.47kg), and 2.37kg (0.11-4.53kg) in the low flux group (p=0.02).

The inclusion criteria were dialysis treatment for at least 4 weeks and absence of any acute clinical event (e.g. myocardial infarction, stroke) or inflammation at the time of recruitment.

The characteristics of these patients and the treatment conditions are presented in Table 2. All patients received multivitamin supplements on routine basis, after haemodialysis and after collection of post-dialysis blood samples. These preparations usually contained water-soluble vitamins (folic acid 1.0mg; pyridoxal-5'-phosphate 10.0mg; cobalamin 6.0µg; thiamine 3.0mg; riboflavin 1.7mg or folic acid 0.16mg; pyridoxal-5'-phosphate 10.0mg; thiamine 8.0mg; riboflavin 8.0mg; furthermore in both preparations ascorbic acid; biotin; pantothenic acid and nicotinic acid amide).

The study protocol was designed in accordance with the declaration of Helsinki and written informed consent had been obtained in all cases.

Measurements

Nonfasting, pre- and post-dialysis blood samples (11.5mL each) were collected in tubes containing EDTA or no additive. Pre-dialysis blood samples were taken immediately before dialysis started from the arterial shunt needle. Post-dialysis blood samples were taken directly after the end of dialysis from the arterial shunt needle. All samples, except for the samples for the thiamine and riboflavin determinations, were immediately cooled and centrifuged, and the plasma or serum was separated off within 30 minutes and frozen at -20°C until analysis. Lysates of EDTA blood in ascorbic acid solution lysis reagent (Elecsys RBC Folate, Roche Diagnostics, Mannheim, Germany) were prepared for analysis of red blood cell (RBC) folate according to the manufacturer's instructions and frozen at -20°C until the time of analysis.

Routine clinicochemical variables were measured by standardized methods on AutoAnalyzers (Hitachi 747, Roche Diagnostics). Plasma tHcy was determined in EDTA plasma by HPLC with fluorescence detection.[16] The upper limit of the reference range for tHcy is 15µmol/L. Serum cobalamin and serum folate were analysed using commercial test kits (Elecsys Module E170, Roche Diagnostics). The test kit measures total cobalamin in the serum. Folate was analysed both in serum and in RBCs. The lower limit of the reference range is 181µg/L for serum cobalamin, 3.1µg/L for serum folate, and 176µg/L for RBC folate. Only patients with serum folate<60µg/L and serum cobalamin<2000ng/L were included, since these are the technical cut-offs of the assay after dilution.

Thiamine and total riboflavin were determined in EDTA whole blood samples by HPLC (Chromsystems, Munich, Germany). Total riboflavin was calculated from flavin adenine dinucleotide (FAD), flavin mononucleotide (FMN), and free riboflavin. The lower limit of the reference range for thiamine is 20µg/L and for total riboflavin it is 47µg/L. Pyridoxal-5'-phosphate (PLP) was determined in EDTA plasma by HPLC (ImmuChrom, Bensheim, Germany). The lower limit for plasma PLP is 4.5µg/L.

Statistical analyses

All post-dialysis values were corrected for haemoconcentration due to the dialysis procedure using pre- and post haematocrit values.
RBC folate was measured in whole blood and corrected for serum folate.
The percentage reductions in the vitamin and tHcy values after dialysis were calculated from the formula: $((value_{pre}-value_{post})/value_{pre})*100$.
Data are presented as medians with 5^{th} and 95^{th} percentiles due to non-Gaussian distributions of the vitamin concentrations. Nonparametric statistical tests, the Wilcoxon (two-sided) signed-rank test and the Mann-Whitney U-test were used for the data analyses. Correlations were measured by Spearman's rank correlation coefficient. Discrete variables were compared by the χ-squared test. The significance threshold was set at $p=0.05$; all tests were two-sided. All the analyses were carried out using the SPSS package Version 9.0 (SPSS Software, Chicago, IL, USA).

Results

There were no differences between the high-flux and low-flux groups in gender, Kt/V_{urea} and duration of the dialysis sessions. However, the patients in the two groups differed significantly with respect to age and months of dialysis treatment (Table 2).

Table 3 shows the blood concentrations of all vitamins measured and of tHcy before and after dialysis.

Table 2: Characteristics of ESRD patients; the data are percentages of patients in each treatment group or medians with 5th and 95th percentiles

			Dialyser membrane				
	All patients (n=30)		Low-flux (LF) (n=15)		High-flux (HF) (n=15)		p LF vs. HF
Age	71	(42-81)	75	(42-82)	58	(40-81)	<0.05
Female (%)	37		47		27		0.26
Months on dialysis	43	(7-98)	24	(6-43)	71	(43-99)	<0.01
Duration of dialysis (hrs/week)	12.5	(9.0-16.5)	12.5	(9.0-13.5)	13.5	(9.0-16.5)	0.12
Kt/V_{urea} [§]	1.4	(1.2-1.8)	1.4	(1.2-1.8)	1.4	(1.2-1.8)	0.74

[§] Kt/V_{urea}=urea nitrogen clearance divided by volume of distribution of urea nitrogen

Table 3: Blood concentrations of folate, cobalamin, pyridoxal-5'-phosphate, thiamine, riboflavin, RBC folate, homocysteine, creatinine, urea, and uric acid before and after dialysis; the data are medians with 5th and 95th percentiles

	All patients (n=30)		p (pre- vs. post-dialyis)
Pre-dialysis serum folate (µg/L)	14.3	(6.8-58.4)	<0.01
Post-dialysis serum folate (µg/L)	9.7	(4.9-31.0)	
Pre-dialysis serum cobalamin (ng/L)	562	(373-1230)	0.09
Post-dialysis serum cobalamin (ng/L)	590	(374-1315)	
Pre-dialysis plasma PLP (µg/L) [§]	12.7	(2.8-46.4)	<0.01
Post-dialysis plasma PLP (µg/L) [§]	7.2	(2.3-29.9)	
Pre-dialysis blood thiamine (µg/L)	80	(57-111)	<0.01
Post-dialysis blood thiamine (µg/L)	74	(53-129)	
Pre-dialysis blood riboflavin (µg/L)	163	(93-324)	<0.01
Post-dialysis blood riboflavin (µg/L)	156	(101-228)	
Pre-dialysis RBC folate (µg/L) [§]	1142	(512-2875)	0.17
Post-dialysis RBC folate (µg/L) [§]	1064	(519-3176)	
Pre-dialysis plasma tHcy (µmol/L) [§]	23.2	(13.5-37.9)	<0.01
Post-dialysis plasma tHcy (µmol/L) [§]	13.1	(7.5-25.1)	
Pre-dialysis serum creatinine (µmol/L)	877	(268-1205)	<0.01
Post-dialysis serum creatinine (µmol/L)	281	(88-436)	
Pre-dialysis serum urea (mmol/L)	24.4	(12.8-34.5)	<0.01
Post-dialysis serum urea (mmol/L)	6.21	(2.84-9.32)	
Pre-dialysis serum uric acid (µmol/L)	439	(335-595)	<0.01
Pre-dialysis serum uric acid (µmol/L)	102	(58-148)	

[§] PLP=pyridoxal-5'-phosphate

RBC=red blood cell

tHcy=total homocysteine (sum of free and protein-bound homocysteine)

We found significantly lower vitamin concentrations after dialysis except for cobalamin and RBC folate, irrespective of dialyser type (Table 4). A significant difference between low-flux and high-flux membranes was only found for tHcy: the reduction in plasma tHcy was greater in the patients on high-flux dialysis (48% vs. 37%; p<0.01). The homocysteine levels fell from 22.7 [13.5-43.7] to 14.6 [7.6-27.6] µmol/L in the low-flux group and from 23.6 [12.4-37.9] to 11.8 [6.1-20.4] µmol/L in the high-flux group.

Table 4: Percentage changes in serum folate, plasma pyridoxal-5'-phosphate, blood thiamine, blood riboflavin, and plasma homocysteine in patients treated with low-flux (n=15) or high-flux dialyser (n=15) during the dialysis process

	Dialyser membrane		p
	Low-flux (LF)	High-flux (HF)	LF vs. HF
Serum folate	- 38%	- 36%	0.68
Plasma PLP §	- 37%	- 29%	0.25
Blood thiamine	- 4%	- 9%	0.19
Blood riboflavin	- 7%	- 6%	0.97
Plasma tHcy §	- 37%	- 48%	< 0.01

§ PLP=pyridoxal-5'-phosphate

tHcy=total homocysteine (sum of free and protein-bound homocysteine)

Correlations were found between the pre-dialysis blood concentrations and intradialytic percentage change for PLP (r=0.531; p<0.01), riboflavin (r=0.616; p<0.01), and folate (r=0.786; p<0.01) (Table 5).

Table 5: Correlations between the blood concentration of folate, pyridoxal-5'-phosphate, thiamine, riboflavin, and homocysteine before dialysis and the percentage change during dialysis for all patients

Correlation	Percentage change	p
Pre-dialysis serum folate (µg/L)	0.786	<0.01
Pre-dialysis plasma PLP (µg/L) §	0.531	<0.01
Pre-dialysis blood thiamine (µg/L)	0.232	0.22
Pre-dialysis blood riboflavin (µg/L)	0.616	<0.01
Pre-dialysis plasma tHcy (µmol/L) §	- 0.111	0.56

§ PLP=pyridoxal-5'-phosphate

tHcy=total homocysteine (sum of free and protein-bound homocysteine)

Looking for determinants of the intradialytic percentage change in the vitamin concentrations, we identified the vitamin/albumin ratio as an important determinant of this change. There was a strong linear association between the folate/albumin ratio and the intradialytic percentage change

in serum folate (r=0.782; p<0.001). Such an association was also observed for PLP (r=0.557; p<0.01) (Figure 1).

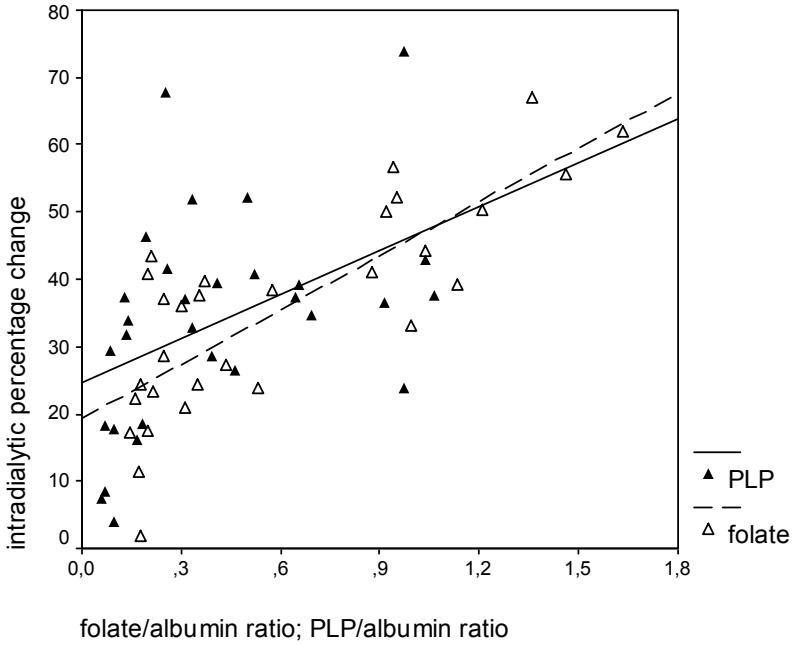

Figure 1: Correlation between the serum folate/serum albumin and plasma pyridoxal-5'-phosphate (PLP)/serum albumin ratios, and corresponding percentage change during dialysis process (r=0.782; p<0.001 for folate and r=0.557; p<0.01 for PLP)

We then compared the percentage changes in vitamin concentrations with the molecular weights of the respective vitamins. For this comparison we used all forms of riboflavin separately (FMN, FAD, free riboflavin) and assumed RBC folate to consist of septaglutamate-methyltetrahydrofolate. The molecular weight of the serum folate was calculated as for monoglutamate-methyltetrahydrofolate. For the analytes measured we observed a strong inverse correlation between the molecular weight and the percentage change after dialysis (r=-0.867; p<0.01) (Figure 2).

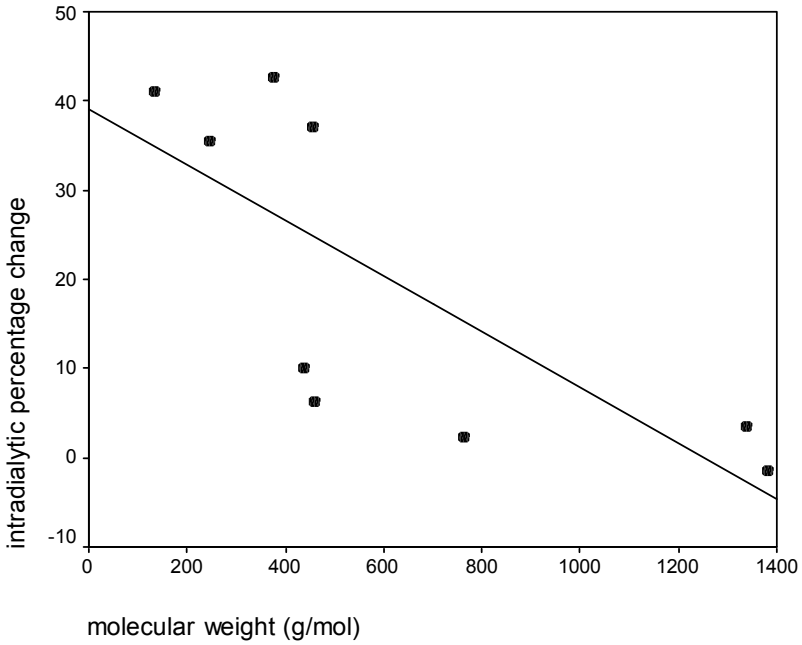

Figure 2: Correlation between the molecular weight of the analyte (serum folate, serum cobalamin, plasma pyridoxal-5'-phosphate (PLP), blood thiamine, blood riboflavin, red blood cell (RBC) folate, plasma homocysteine (tHcy)), and corresponding percentage change during dialysis ($r=-0.867$; $p<0.01$)

Discussion

The aim of this study was to investigate the intradialytic loss of vitamins and of homocysteine in patients with end-stage renal disease, and to establish whether this loss is dependent on the dialyser type. The main finding is that vitamins are indeed lost during dialysis, but the type of dialyser membrane is not important in the case of the vitamins. In contrast, the homocysteine reduction is significantly more efficient in the case of the high-flux dialysers. The reduction in vitamins is determined mainly by the vitamin concentration prior to the dialysis, the molecular weight of the vitamin, and the pre-dialysis vitamin/albumin ratio.

We investigated the effects of dialysis on serum and RBC folate, serum cobalamin, plasma PLP, blood thiamine, blood riboflavin, and homocysteine. The molecular weights of these compounds are in the range of 135-1400 Dalton, which is typical for so called small-to-middle molecules. Since many other molecules in this size range are regarded as uraemic toxins,[17] their reduction by dialysis is desirable; thus, the washout of vitamins is a consequence of efficient dialysis, which has not demonstrated earlier.

We also looked for more specific vitamin loss determinants, and identified the folate/albumin ratio and the PLP/albumin ratio as important factors influencing the intradialytic percentage change. To our knowledge, this is a new finding. The non-specific binding of folate and PLP to serum albumin has been demonstrated long ago,[18,19] and in the case of folate it can make up to 50% of the total folate.[18] It cannot be said at present whether the occurrence of unmetabolized folic acid in serum, which can be observed after high-dose supplementation with folic acid,[20,21] contributes to this phenomenon.

At present water-soluble vitamins are not prescribed in ESRD patients on a routine basis, and there is a large variation in the prescription of vitamins between different countries.[6] Recommendations for higher dialysis doses[22] and the use of more permeable dialyser membranes with larger surfaces may increase the losses of micronutrients. In particular, we found that serum folate and plasma PLP were reduced during dialysis (37% and 35%). A slight decrease was also observed in the blood concentrations of thiamine (6%) and riboflavin (7%). High losses of folate and PLP have similarly been reported by Leblanc.[23] As expected, cobalamin and RBC folate were not reduced by dialysis.

We did not observe any dialyser membrane effect, in contrast to investigations at long-term effects of high-flux dialysis. Kasama[14] investigated the effect of high-flux membranes over 1 month or longer, and observed a decrease in plasma PLP and in serum folate by about 50% during this period. Serum cobalamin and RBC folate levels were not influenced by long-term treatment with high-flux membranes.[11] Further studies are evidently needed to clarify the long-term effect of high-flux membranes on vitamin homeostasis.

The reduction of homocysteine levels by dialysis has been reported by many authors.[11-13,24] However, we are not aware of studies showing that patients dialysed with high-flux membranes have lower pre-dialysis homocysteine concentrations than patients dialysed with low-flux membranes. It is known that plasma homocysteine rises immediately after dialysis,[24] and the decrease in plasma folate due to high-flux membranes over a longer period may contribute to the elevation of homocysteine.[11] Significant lower pre-dialysis level of tHcy were only observed in patients treated with super-flux for 3 to 6 months compared to patients treated with standard high-flux dialysis, probably because of the removal of uraemic toxins with inhibitory activities against enzymes involved in the extra-renal homocysteine metabolism.[25,26] Also treatment with nocturnal haemodialysis is independently associated with lower tHcy levels before dialysis.[27] However, the use of FX-class dialysers, which have a better clearance of larger uraemic toxins

than standard high-flux dialysers but are non-albumin-leaking, did not result in a clinically significant benefit with regard to improved pre-dialysis concentrations of tHcy.[28]

Although this is a non-randomized trial with a relative small number of patients in the treatment groups, which are not balanced regarding age and time on dialysis it can be summarized, that the blood concentrations of water-soluble vitamins are reduced by the dialysis procedure. The nature of the dialysis membranes, high-flux or low-flux, has no influence on washout of vitamins. Monitoring of vitamin status of patients with end-stage renal disease is necessary regardless whether they are treated with high flux or low flux dialysers. Long term studies will add to our understanding of the influence of the dialysis procedure on vitamin metabolism.

References

1. Foley RN, Parfrey PS, Sarnak MJ. Epidemiology of cardiovascular disease in chronic renal disease. J. Am Soc Nephrol. 1998; 9:S16-S23.
2. Ritz E, Koch M. Morbidity and mortality due to hypertension in patients with renal failure. Am J Kidney Dis. 1993; 21:S113-S118.
3. Baigent C, Wheeler D, Burbury K. Premature cardiovascular disease in chronic renal failure. Lancet 2000; 356:147-152.
4. Zimmermann J, Herrlinger S, Pruy A et al. Inflammation enhances cardiovascular risk and mortality in hemodialysis patients. Kidney Int. 1999; 55:648-658.
5. Owen WF. Nutritional status and survival in end-stage renal disease patients. Miner Electrolyte Metab. 1998; 24:72-81.
6. Fissell RB, Bragg-Gresham JL, Gillespie BW et al. International variation in vitamin prescription and association with mortality in the Dialysis Outcomes and Practice Patterns Study (DOPPS). Am J Kidney Dis. 2004; 44:293-299.
7. Fouque D, Vennegoor M, ter Wee P et al. EBPG guideline on nutrition. Nephrol Dial Transplant. 2007; 22:ii45-ii87.
8. Domröse U, Heinz J, Westphal S, Luley C, Neumann KH, Dierkes J. Vitamins are associated with survival in patients with end-stage renal disease: a 4-year prospective study. Clin Nephrol. 2007; 4:221-229.
9. Jamison RL, Hartigan P, Kaufman JS et al. Effect of homocysteine lowering on mortality and vascular disease in advanced chronic kidney disease and end-stage renal disease: a randomized controlled trial. JAMA 2007; 298:1163-1170.
10. Cheung AK, Levin NW, Greene T et al. Effects of high-flux hemodialysis on clinical outcomes: results of the HEMO study. J Am Soc Nephrol. 2003; 14:3251-3263.
11. Lasseur C, Parrot F, Delmas Y et al. Impact of high-flux/high-efficiency dialysis on folate and homocysteine metabolism. J Nephrol. 2001; 14:32-35.

12. De Vriese AS, Langlois M, Bernard D et al. Effect of dialyser membrane pore size on plasma homocysteine levels in haemodialysis patients. Nephrol Dial Transplant. 2003; 18:2596-2600.
13. House AA, Wells GA, Donnelly JG, Nadler SP, Hebert PC. Randomized trial of high-flux vs low-flux haemodialysis: effects on homocysteine and lipids. Nephrol Dial Transplant. 2000; 15:1029-1034.
14. Kasama R, Koch T, Canals-Navas C, Pitone JM. Vitamin B6 and hemodialysis: the impact of high-flux/high-efficiency dialysis and review of the literature. Am J Kidney Dis. 1996; 27:680-686.
15. Bostom AG, Culleton BF. Hyperhomocysteinemia in chronic renal disease. J Am Soc Nephrol. 1999; 10:891-900.
16. Vester B, Rasmussen K. High performance liquid chromatography method for rapid and accurate determination of homocysteine in plasma and serum. Eur J Clin Chem Clin Biochem. 1991; 29:549-554.
17. Yavuz A, Tetta C, Ersoy FF et al. Uremic toxins: a new focus on an old subject. Semin Dial. 2005; 18:203-211.
18. Soliman HA, Olesen H. Folic acid binding by human plasma albumin. Scand J Clin Lab Invest. 1976; 36:299-304.
19. Lumeng L, Brashear RE, Li TK. Pyridoxal 5'-phosphate in plasma: source, protein-binding, and cellular transport. J Lab Clin Med. 1974; 84:334-343.
20. Kelly P, McPartlin J, Goggins M, Weir DG, Scott JM. Unmetabolized folic acid in serum: acute studies in subjects consuming fortified food and supplements. Am J Clin Nutr. 1997; 65:1790-1795.
21. Ghandour H, Bagley PJ, Shemin D et al. Distribution of plasma folate forms in hemodialysis patients receiving high daily doses of L-folinic or folic acid. Kidney Int. 2002; 62:2246-2249.
22. NKF-DOQI Clinical Practice Guidelines for Hemodialysis Adequacy. National Kidney Foundation, New York, NY: 1997; 41-46.
23. Leblanc M, Pichette V, Geadah D, Ouimet D. Folic acid and pyridoxal-5'-phosphate losses during high-efficiency hemodialysis in patients without hydrosoluble vitamin supplementation. J Ren Nutr. 2000; 10:196-201.
24. Arnadottir M, Wingren K, Hultberg B, Hegbrant J. The postdialytic rise in the plasma total homocysteine concentration is delayed. Blood Purif. 2002; 20:334-337.
25. Van Tellingen A, Grooteman MP, Bartels PC et al. Long-term reduction of plasma homocysteine levels by super-flux dialyzers in hemodialysis patients. Kidney Int. 2001; 59:342-347.
26. Galli F, Benedetti S, Buoncristiani U et al. The effect of PMMA-based protein-leaking dialyzers on plasma homocysteine levels. Kidney Int. 2003; 64:748-755.
27. Friedman AN, Bostom AG, Levey AS, Rosenberg IH, Selhub J, Pierratos A. Plasma total homocysteine levels among patients undergoing nocturnal versus standard hemodialysis. J Am Soc Nephrol. 2002; 13:265-268.
28. Mudge DW, Rogers R, Hollett P et al. Randomized trial of FX high flux vs standard high flux dialysis for homocysteine clearance. Nephrol Dial Transplant. 2005; 20:2178-2185.

4

Vitamins are Associated with Survival in Patients with End-Stage Renal Disease: a 4-year Prospective Study

Based on the original article:

Domröse U, Heinz J, Westphal S, Luley C, Neumann KH, Dierkes J. Vitamins are associated with survival in patients with end-stage renal disease: a 4-year prospective study. Clin Nephrol. 2007; 67:221-229.

Abstract

Background:

Patients with end-stage renal disease are at high risk from premature death due mainly to cardiovascular disease and infections. Established risk factors do not sufficiently explain this increased mortality. We therefore investigated total mortality prospectively in a single-centre study in patients on haemodialysis and assessed the prognostic value of baseline disease status, laboratory variables including emerging risk factors, and the influence of vitamin treatment.

Methods:

Patients (n=102) were followed up for 4 years or until death (n=49). Survival was calculated by the Kaplan-Meier method. Cox proportional hazards model was used to determine independent predictors of total mortality.

Results:

The known risk factors of age, baseline clinical atherosclerotic disease, low albumin, and increased cardiac troponin T were significantly associated with mortality. Patients who received multivitamins during follow-up had a significantly lower mortality risk than those not receiving this treatment (hazard ratio 0.29, 95% confidence interval 0.15-0.56). These associations remained significant after adjustment for age, cardiovascular disease, albumin, and cardiac troponin T at baseline.

Conclusions:

The present study suggests that multivitamin supplementation in patients with end-stage renal disease is closely associated with reduced mortality due to all causes. These observations have to be validated in randomized clinical intervention trials.

Key words: end-stage renal disease, total mortality, troponin, vitamins

Abbreviations:

tHcy	Total homocysteine (sum of free and protein-bound homocysteine)
cTnT	Cardiac troponin T
Lp(a)	Lipoprotein(a)
sICAM-1	Soluble Intercellular adhesion molecule 1
CVD	Cardiovascular disease
ESRD	End-stage renal disease
CI	Confidence interval
HR	Hazard ratio
MV	Multivitamins

Introduction

End-stage renal disease (ESRD), which affects approximately 57,500 patients in Germany and about 1,000,000 patients worldwide,[1-3] is associated with a substantially reduced life expectancy estimated at less than 50% of the life expectancy of a person at the same age but with normal renal function.[4] In particular, ESRD patients have a considerable increase in cardiovascular risk, which contributes to their high mortality rates.[5] Age, underlying diseases such as diabetes mellitus, chronic inflammations, and deprived nutritional status are believed to have a pronounced effect on survival time during haemodialysis therapy.[6-8] Variations in their therapy, such as the type of the dialysis membrane, control of hypertension, or anaemia may additionally contribute to morbidity and mortality.[9,10] Some effects of these differences on morbidity and mortality have been studied in prospective studies, but, the impact of many aspects of therapy on survival has not been investigated, either in prospective observation studies or in intervention studies.

Awareness of nutritional vitamin status in haemodialysis (HD) patients has grown in the last few years. It has been recognized that these patients are at increased risk of water-soluble-vitamin deficiency compared with healthy subjects. This is due to their dietary restrictions leading to decreased vitamin intake, to loss of vitamins during dialysis[11-13] and to increased vitamin demand due to renal failure.[14,15] Treatment with water-soluble vitamin preparations, including folic acid, vitamin B6, and B12 may reduce the elevated homocysteine concentrations frequently observed in ESRD patients,[16-17] improve haematopoiesis or act as antioxidant therapy.[18,19] However, a consensus on whether multivitamins should be prescribed to all ESRD patients has not been achieved, and the percentage of haemodialysis patients receiving multivitamins varies enormously between countries and facilities.[20] Many dialysis patients are given multivitamin supplements on a routine basis without any evaluation of their effects. Although the main reason for prescribing multivitamins is to avoid deficiency, other health aspects believed to occur but are difficult to substantiate.

In the recent Dialysis Outcomes and Practice Patterns Study (DOPPS) treatment with water-soluble vitamins reduced the relative risk of mortality in ESRD patients by 16% (RR 0.84; 95%CI, 0.76-0.94).[20] It is therefore an interesting hypothesis that prescription of water-soluble multivitamins may reduce mortality in ESRD patients. We report here the results of a prospective observation study in a cohort of 102 haemodialysis patients with a follow-up period of 4 years which adds to the knowledge of the use of multivitamins and mortality in ESRD patients. We found that in a single dialysis centre, multivitamin use was associated

with a reduced mortality even after adjustment for important variables such as cardiac troponin T.

Study design

The study design has been described in detail elsewhere.[21] Briefly, patients suffering from ESRD treated by chronic intermittent haemodialysis at the outpatients Dialysis Centre for at least 4 weeks and clinically stable at the time of recruitment, were eligible for inclusion in the study. Out of 112 patients treated by haemodialysis at the centre at entry into the study, 102 (91%) were recruited. The exclusion criteria were age >85 years (n=6) and unstable clinical status at study entry (n=4). Informed consent was obtained from each patient. The prevailing diseases, weight and height, pre-dialysis blood pressure and lifestyle (smoking, use of vitamin supplements, prescribed medication, erythropoietin use) and dialysis factors (duration of dialysis, dialysis membrane, Kt/V according to the double pool method) were recorded at baseline. A non-fasting pre-dialysis blood sample was taken for the determination of clinical-chemical variables (blood count, electrolytes, albumin, protein, creatinine, urea, glucose) and of cardiovascular-risk-associated factors: total cholesterol, HDL-cholesterol, LDL-cholesterol, triglycerides, apolipoprotein B, lipoprotein(a), total homocysteine (tHcy), serum and red blood cell folate, vitamin B12, and cardiac troponin T (cTnT). The results of the clinical-chemical variables and lipids were made available to clinicians in charge of the patients via routine laboratory charts. Cardiac troponin T and lipoprotein(a) were measured in aliquots of serum samples obtained at entry into the study which had been stored for nearly 2 years at -70°C. These and the results of the tHcy and the vitamin determinations, however, were not included in the routine analyses and were therefore not made available to the clinicians, but kept at the laboratory. The individual tHcy, vitamin and cTnT results were not passed on to the clinicians during the 4-year follow-up period.

Follow-up

Patients were followed up for 4 years. After the first 2 years of follow-up, an analysis for the endpoints ‘cardiovascular disease’ and ‘total mortality’ was carried out.[21] After 4 years the outcome was again recorded by nephrologists at the Dialysis Centre, who were unaware of the individual results for tHcy, vitamins, cTnT, and Lp(a). If a patient had moved to another

dialysis centre, information about death was obtained from the dialysis centre at which the patient had been treated (9 patients). 10 patients received a renal transplant, and these patients were followed up until the date of the transplantation and then censored. None of the transplantation patients died during the follow-up.

Baseline cardiovascular disease was considered if the patient had suffered a proven clinical event (myocardial infarction, stroke, ulcers or surgery for peripheral vascular disease) or angiographical evidence of relevant stenosis of the respective artery (coronary, carotid or femoral). Hypertension was defined either as use of anti-hypertensive drugs or a pre-dialysis blood pressure exceeding 160/95mmHg.

The quality of the treatment with dialysis was regularly monitored using Kt/V recorded by the physician every 6 months.

In the event of death, the underlying cause and the date of death were recorded. Causes of death were classified into cardiovascular disease (sudden death, myocardial infarction, heart failure, stroke, peripheral arterial disease), infections (pneumonia, sepsis, viral infections), tumours, and other causes. In 9 of the 49 deaths (18%), the underlying cause of death was verified by autopsy. Due to the high percentage of unknown causes of deaths (22%), no further analysis was carried out with respect to cause of death.

Treatment and other diseases during follow up

The patients' records were reviewed for treatment changes during follow-up, hospitalizations, vitamin supplementation, and infections requiring specific treatment. Special attention was paid to multivitamin treatment with water-soluble vitamins. Multivitamins were not given routinely to each patient. The decision to prescribe vitamin supplementation was taken by the clinician in charge of the patients, using preparations specifically designed for patients with end-stage renal disease. These preparations usually contained water-soluble vitamins (folic acid 1000µg; pyridoxal-5'-phosphate 6000µg; cobalamin 6.3µg; furthermore ascorbic acid; thiamine; riboflavin; biotin; pantothenic acid and nicotinic acid amide). The prescribed multivitamins were usually administered after each dialysis session to ensure compliance (usually 3 times per week) for a median time of 3 months.

Statistical analysis

Values are presented as means with their standard deviations or as medians with 5th and 95th percentiles. In a first analysis, differences at baseline were investigated between the patients who died and those who were alive after 4 years, using the Mann-Whitney U-test for skewed variables and Student's t-test for normally distributed variables. Discrete variables were compared by the Chi-squared test. Observed survival was calculated by the Kaplan-Meier method. Cox proportional-hazards regression analysis was used to examine the baseline variables predictive of total mortality. The models included the variables that showed significant differences in univariate analyses. Adjustments were then made for the baseline variables considered *a priori* to be important predictors of mortality: age, albumin, cardiovascular disease and cTnT.

Age and albumin were added as continuous variables, while cardiovascular disease (yes/no) and cTnT (0.05 as cut off level) were introduced into the model as a categorized variable. Cardiac TnT level lower than 0.05 was observed in 71 patients, and cTnT level of 0.05ng/ml or higher was measured in 31 patients. Results are reported as hazard ratios (HR) with respective 95% confidence intervals (CI). The significance threshold was set at p=0.05; all tests were two-sided. All analyses were carried out using SPSS Version 9.0.

Analytical methods

The laboratory methods have been described previously.[21] Standard methods were used for clinical-chemical variables (Hitachi 747, Roche Diagnostics, Mannheim, Germany), blood count, vitamins, lipids, and cTnT. Plasma tHcy was determined in EDTA-plasma at entry into the study using an HPLC method with fluorescence detection.[22] The total homocysteine levels exceeded the upper reference limit (15 μmol/L) in 100 of the 102 (98%) patients. Folate levels below the lower reference limit were not observed.

The cTnT concentration was measured in aliquots of serum samples stored at -70°C for nearly 2 years, using a second-generation test system with improved sensitivity (Roche Diagnostics, Mannheim, Germany). The upper reference limit of this test, 0.04ng/mL, was exceeded in 40 patients (38%). Cardiac troponin T was above the limit of detection (0.01ng/ml) in 85 patients (83%).

Blinding

The laboratory staff and researchers remained unaware of the patients' baseline clinical status until the end of the study. The clinical data including the patients' baseline and outcome status were recorded by the physicians, who were unaware of the laboratory results of tHcy, vitamins, cTnT, and Lp(a) determinations. Blinding was accomplished by keeping the patients' clinical data files separately from the laboratory results files until the study had been completed.
Data concerning the vitamin treatment and hospitalizations were collected at the end of the 4-year study period from patients' records by a nephrologist not involved in the statistical analyses.

Results

Out of the 102 patients included, 37 (36%) received multivitamin preparations during follow-up for a median period of 6 months. The patients receiving multivitamins did not differ significantly at the start of the study from those receiving no vitamins with respect to age, blood pressure, existing baseline diseases, and most of the clinical chemical variables (Table 1). However, those receiving multivitamins had significantly lower levels of cardiac troponin T and sICAM-1.
During the follow-up period of 4 years, 49 patients (21 men and 28 women) died. The main cause of death was CVD, occurring in n=17 patients (35%), the next most frequent cause was infection, which occurred in n=14 patients (29%). The frequency of other causes (e.g. acute pancreatitis, discontinuation of dialysis) was n=7 (14%), and in n=11 patients (22%) the cause of death could not be ascertained.

Table 1: Baseline characteristics of haemodialysis patients (n=102, representing 92% of all patients treated at the dialysis centre). In total, 50 men and 52 women were included. Data are given as numbers and percentages of patients in each treatment group, or as means ± standard deviations or medians with 5th and 95th percentiles (MV=multivitamins)

		Patients receiving no MV during follow-up (n=65)	Patients receiving MV during follow-up (n=37)	p
Age at baseline	mean±SD	65±13	62±14	0.32
Duration of dialysis (months)	median (5^{th}-95^{th} perc.)	25 (1-97)	25 (6-78)	0.89
Systolic blood pressure	mmHg mean±SD	133±17	136±14	0.39
Diastolic blood pressure	mmHg mean±SD	76±9	78±7	0.44
Patients who have ever smoked (n = 101)	n (%)	28 (44%)	17 (46%)	0.84
Baseline disease:				
Patients with diabetes	n (%)	31 (48%)	12 (32%)	0.13
Patients with baseline atherosclerotic disease	n (%)	30 (46%)	14 (38%)	0.42
Treatment at Baseline:				
Haematocrit		0.31±0.03	0.32±0.03	0.58
Erythropoietin	n (%)	49 (75%)	28 (76%)	0.92
Kt/V	mean±SD	1.55±0.36	1.50±0.28	0.45
Ca x P product	$mmol^2/L^2$ mean±SD	4.21±1.37	4.22±0.95	0.96
Clinical-chemical variables at baseline:				
Total cholesterol	mmol/L mean±SD	5.69±1.34	5.75±1.25	0.94
Triglycerides	mmol/L mean±SD	3.12±2.51	3.42±3.27	0.52
Serum cobalamin	pg/mL mean±SD	549±481	479±345	0.99
Serum folate	ng/mL mean±SD	9.22±4.20	9.38±3.11	0.42
Total homocysteine	µmol/L mean±SD	39.2±17.0	33.0±14.6	0.05
Cardiac troponin T	ng/ml mean±SD	0.063±0.069	0.026±0.019	<0.01
sICAM-1	ng/ml mean±SD	267±108	212±66	<0.01

We calculated hazard ratios for total mortality by Cox regression modelling. In the univariate Cox regression model, albumin, sICAM-1, cTnT, baseline CVD, diabetes, use of a high-flux membrane, and multivitamin use were significantly associated with total mortality (Table 2). After adjustment for factors selected *a priori* (albumin, CVD, and age), diabetes mellitus and the dialysis membrane were no longer significantly associated with mortality (Table 2).

A large increase in risk was associated with the baseline cTnT. For cTnT values>0.1 ng/ml the crude hazard ratios were 5.69; 95% CI, 2.76-11.68, decreased to 2.75; 95% CI, 1.18-6.42 after adjustment for age, baseline cardiovascular disease, and albumin. This is in accord with a number of other studies in patients with ESRD and will not be discussed further.[21,23,24]

At baseline, tHcy was slightly increased in patients receiving no multivitamins compared with patients receiving multivitamin treatment (39.2±17.0µmol/L vs. 33.0±14.6µmol/L; p=0.05). However, in the univariate Cox regression model tHcy showed no association with mortality (HR 1.22; 95% CI, 0.65-2.26).

Soluble ICAM-1 was also significantly different in patients receiving multivitamins compared to those who did not receive this treatment. In the Cox regression, sICAM-1 was also associated with mortalilty, even after adjustment for cTnT (Table 2).

Treatment with water-soluble vitamins had a profound effect on mortality. Only 30% of the patients receiving multivitamins died as opposed to 59% of those who did not, leading to a crude HR of 0.29; CI 95%, 0.15-0.56. The association remained significant after adjustment for age, CVD, albumin, and cTnT (Table 2). Further adjustments for the calcium-phosphate product, diabetes mellitus, baseline folate, tHcy, or sICAM-1 did not change these results (data not shown).

Figure 1 shows the Kaplan-Meier curves obtained from a Cox regression analysis for patients with and without multivitamin treatment. The treatment was significantly associated with improved survival (p<0.01). A further adjustment for history of diabetes mellitus had no substantial effect on the results (data not shown).

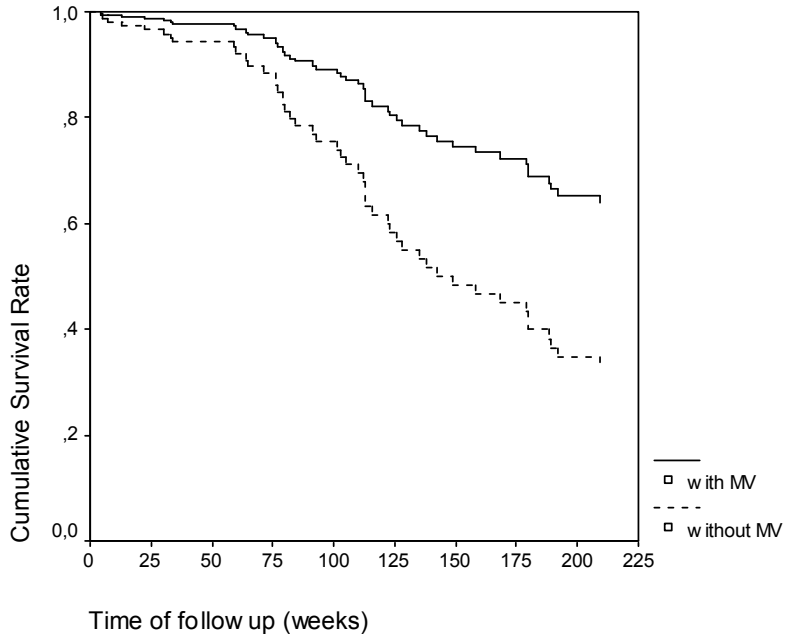

Figure 1: Estimated survival rate for 102 patients with end-stage renal disease according to multivitamin use during follow-up. The follow-up time was 4 years. 10 patients were censored because of transplantation during follow-up. The figure shows estimated survival rate adjusted for age, albumin, cardiovascular disease, and cTnT concentrations at baseline. The survival rate curves were calculated using a Cox regression analysis (MV=multivitamins). Crude HR with 95% CI for multivitamin use yes/no: 0.29(0.15-0.56)

Table 2: Baseline disease status and laboratory variables of ESRD patients according to the outcome variable 'total mortality'. The values given are medians with 5th and 95th percentiles. Significance was calculated by the Mann-Whitney U-test or the chi-squared test.
Hazard ratios during a follow-up period of 4 years in 102 patients with ESRD treated by hemodialysis, calculated by Cox regression analysis for outcome variable 'total mortality', based on 49 deaths.

		Patients alive n=53	**Patients dead n=49**	**p**	**Risk calculated for**	**Crude HR with 95% CI**	**Adjusted# HR with 95% CI**	**Adjusted* HR with 95% CI**
Age		59 ± 14	69 ± 9	<0.001	per year ↑	1.05 (1.02-1.08)	1.03 (0.99 -1.06)	1.03 (0.99-1.07)
Baseline cardio-vascular disease		14 (26%)	30 (41%)	0.01	yes=1, no=0	2.66 (1.49-4.73)	1.85 (1.01-3.40)	1.69 (0.90-3.20)
Diabetes mellitus	n (%)	16 (30%)	27 (55%)	0.02	yes=1, no=0	2.00 (1.14-3.52)	1.47 (0.82-2.66)	1.34 (0.74-2.45)
Clinical-chemical risk factors in ESRD patients:								
Serum albumin	g/L	44.0 ± 2.3	41.4 ± 4.5	<0.001	2 g/L ↑	0.41 (0.30-0.56)	0.71 (0.61-0.83)	0.75 (0.64-0.88)
tHcy	µmol/L	32.4 (19.8-78.8)	34.5 (19.5-63.6)	0.59	5 µmol/L ↑ >45 µmol/L=1	1.02 (0.83-1.26) 1.22 (0.65-2.26)	1.10 (0.99-1.23) 1.92 (0.99-3.70)	1.08 (0.96-1.20) 1.79 (0.91-3.52)
Serum folate	nmol/L	9.1 (5.5-13.6)	7.6 (5.2-19.2)	0.06	5 nmol/L ↑	0.73 (0.26-2.13)	0.92 (0.58-1.45)	1.13 (0.71-1.85)
Serum cobalamin	pg/mL	363 (204-829)	437 (202-2000)	0.10	100 pg/mL ↑	1.20 (1.06-1.37)	1.07 (0.99-1.14)	1.08 (1.01-1.14)
sICAM-1	ng/mL	222 ± 72	275 ± 115	0.02	50 ng/mL ↑	1.20 (1.07-1.35)	1.16 (1.03-1.33)	1.14 (1.01-1.30)
Cardiac troponin T	ng/mL	0.019 (<0.01-0.096)	0.042 (0.011-0.172)	<0.001	> 0.05ng/mL =1 > 0.10ng/ml =1	2.72 (1.52-4.88) 5.69 (2.76-11.68)	2.15 (1.15-4.04) 2.75 (1.18-6.42)	---- ----
Dialysis related variables:								
High-flux membrane	n (%)	46 (87%)	31 (63%)	0.01	yes=1, no=0	0.39 (0.22-0.69)	0.59 (0.31-1.14)	0.56 (0.30-1.06)
Multivitamin use during follow-up	n (%)	26 (47%)	11 (22%)	0.01	yes=1, no=0	0.29 (0.15-0.56)	0.36 (0.18-0.72)	0.39 (0.19-0.79)

Discussion

In this prospective study a profound inverse association was observed between treatment with multivitamins and long-term mortality in patients with end-stage renal disease. The association remained also significant after adjustment for known risk factors, like age, pre-existing cardiovascular disease, for cTnT, a strong predictor of mortality and for variables associated with inflammation and/or nutritional status (sICAM-1 and albumin). The mechanisms underlying the possible effect of vitamins on mortality may be manifold, since vitamins are chemically distinct substances.

One possible mode of action of folic acid in lowering the risk of morbidity and mortality is reduction of the raised blood concentrations of tHcy,[14,25-27] encountered in more than 85% of patients with renal failure.[15] It was in fact been shown that in haemodialysis patients higher tHcy levels were associated with increased risk of non-fatal CVD, fatal CVD and total mortality.[28-30,21] The homocysteine-lowering action of multivitamins is due mainly to the folic acid component.[27,31] A further beneficial effect of folic acid may be the improvement of endothelial dysfunction observed after its acute or chronic administration, since endothelial dysfunction is a risk factor for cardiovascular disease.[32] However, even though folic acid has been shown to improve endothelial function in healthy humans,[33] this was not observed in patients with renal insufficiency.[34-36] Another potential beneficial effect of folic acid and vitamin B12 may occur via prevention of anaemia due to their haematopoietic properties. Anaemia is frequent in dialysis patients and has been associated with increased risk of heart failure and death.[5]

In the case of vitamin C, its antioxidative potential must be considered as well as effects on endothelial function.[37] The results of a recent prospective study showed that low total vitamin C plasma levels predicted cardiovascular morbidity and mortality among haemodialysis patients.[38]

Furthermore, haemodialysis is a protein catabolic procedure. It is combined with dialysate amino acid loss and reduced protein synthesis followed by protein malnutrition characterized by losses of somatic protein stores and visceral protein concentration (reflected by lean body mass and albumin).[39,40] A large number of studies have demonstrated that the presence of uraemic malnutrition increases mortality and morbidity in patients treated with haemodialysis.[41,42] Finally, vitamin B6 affects mainly the metabolism of amino acids and proteins. Pyridoxal-5'-phosphate, the active form of vitamin B6, acts as a coenzyme in all transaminations, and in some decarboxylation and dehydration reactions of amino acids.

Losses of vitamin B6 and vitamin C during dialysis are well documented,[11,13] so that prevention of a low vitamin status may also be achieved by supplementation. For other vitamins data are lacking concerning their effect on overall health, although mechanisms may be operating. As an example, a deficiency of thiamine may affect the nervous system and may contribute to neuropathy, a condition which is common in patients with ESRD.[43,44] In addition, nicotinic acid, widely used for treatment of lipid disorders, may reduce long-term mortality in patients with coronary artery disease and may slow or reverse the progression of atherosclerosis, although usually much higher doses are required than provided by a multivitamin pill.[45,46]

Because of the heterogeneity of the preparations used, the data do not allow any conclusions on the contribution of the individual components. The specific effect of each vitamin requires further research in patients with end-stage renal disease. In our view, the primary candidates are folic acid, vitamin B6, and vitamin C.

To the best of our knowledge, there has only been one other study to date on the effect of multivitamins on mortality in patients treated by HD. This was the multi-centre DOPPS study, which was performed in 7 countries in adult HD patients. In DOPPS, a significant 16% reduction in total mortality was observed in patients receiving multivitamin preparations.[20] Thus, in the first phase, our results confirm the DOPPS data. Secondly, our data shows that the risk reduction was sustained even after adjustment for the strong risk factor cardiac troponin T.

An observation study can bring to light associations, but cannot prove a hypothesis. Owing to its design, our study has certain limitations that must be taken into account. The study was not randomized, and was therefore subject to bias. It cannot be excluded that prescription of multivitamins is also a marker of more meticulous overall care of the patient, although most baseline data were similar. Misclassification of over-the-counter multivitamins may have occurred, though this lead to an underestimation of the beneficial action of vitamins.

Bearing that above limitations in mind, these results and the DOPPS data suggest that a randomized clinical trial with multivitamins in haemodialysis patients would be justified to confirm the hypothesis. Patients treated by haemodialysis are characterized by an excessive burden of cardiovascular disease and a high risk of mortality, which are not explained sufficiently by established risk factors. Moreover, well known cardiovascular risk factors behave quite differently in patients with ESRD.[47] For example, an inverse relationship between cholesterol and mortality was found as a marker for malnutrition,[48] and blood

pressure has a U-shaped relationship with mortality.[49] Based on these data, specific intervention trials in dialysis patients aimed at investigating the clinical benefits of multivitamin treatment have been initiated.[50,51]

References

1. Frei U, Schober-Halstenberg HJ. Annual Report of the German Renal Registry 1998. QuaSi-Niere Task Group for Quality Assurance in Renal Replacement Therapy. Nephrol Dial Transplant. 1999; 14:1085-1090.
2. Lysaght MJ. Maintenance dialysis population dynamics: current trends and long-term implications. J Am Soc Nephrol. 2002; 1:S37-S40.
3. Dirks J. A world perspective on renal care: the challenges of prevention and treatment. EDTNA ERCA J. 2005; 31:72-74.
4. Byrne C, Vernon P, Cohen JJ. Effect of age and diagnosis on survival of older patients beginning chronic dialysis. JAMA. 1994; 271:34-36.
5. Foley RN, Parfrey PS, Sarnak MJ. Epidemiology of cardiovascular disease in chronic renal disease. J Am Soc Nephrol. 1998; 9:S16-S23.
6. Baigent C, Wheeler D, Burbury K. Premature cardiovascular disease in chronic renal failure. Lancet. 2000; 356:147-152.
7. Zimmermann J, Herrlinger S, Pruy A, Metzger T, Wanner C. Inflammation enhances cardiovascular risk and mortality in hemodialysis patients. Kidney Int. 1999; 55:648-658.
8. Owen WF. Nutritional status and survival in end-stage renal disease patients. Miner Electrolyte Metab. 1998; 24:72-81.
9. Port FK, Hulbert-Shearon TE, Wolfe RA et al. Predialysis blood pressure and mortality risk in a national sample of maintenance hemodialysis patients. Am J Kidney Dis. 1999; 33:507-517.
10. Foley RN, Parfrey PS, Harnett JD, Kent GM, Murray DC, Barre PE. The impact of anemia on cardiomyopathy, morbidity, and mortality in end-stage renal disease. Am J Kidney Dis. 1996; 28:53-61.
11. Kasama R, Koch T, Canals-Navas C, Pitone JM. Vitamin B6 and hemodialysis: the impact of high-flux/high-efficiency dialysis and review of the literature. Am J Kidney Dis. 1996; 27:680-686.
12. Tamura T, Johnston KE, Bergman SM. Homocysteine and folate concentrations in blood from patients treated with hemodialysis. J Am Soc Nephrol. 1996; 7:2414-2418.
13. Morena M, Cristol JP, Bosc JY et al. Convective and diffusive losses of vitamin C during haemodiafiltration session: a contributive factor to oxidative stress in haemodialysis patients. Nephrol Dial Transplant. 2002; 17:422-427.
14. Jennette JC, Goldman ID. Inhibition of the membrane transport of folates by anions retained in uremia. J Lab Clin Med. 1975; 86:834-843.
15. Livant EJ, Tamura T, Johnston KE et al. Plasma folate conjugase activities and folate concentrations in patients receiving hemodialysis. J Nutr Biochem. 1994; 5:504-508.

16. Bostom AG, Shemin D, Lapane KL et al. High dose-B-vitamin treatment of hyperhomocysteinemia in dialysis patients. Kidney Int. 1996; 49:147-152.
17. Bostom AG, Culleton BF. Hyperhomocysteinemia in chronic renal disease. J Am Soc Nephrol. 1999; 10:891-900.
18. Koury MJ, Ponka P. New insights into erythropoiesis: the roles of folate, vitamin B12, and iron. Annu Rev Nutr. 2004; 24:105-131.
19. Locatelli F, Canaud B, Eckardt KU, Stenvinkel P, Wanner C, Zoccali C. Oxidative stress in end-stage renal disease: an emerging threat to patient outcome. Nephrol Dial Transplant. 2003; 18:1272-1280.
20. Fissell RB, Bragg-Gresham JL, Gillespie BW et al. International variation in vitamin prescription and association with mortality in the Dialysis Outcomes and Practice Patterns Study (DOPPS). Am J Kidney Dis. 2004; 44:293-299.
21. Dierkes J, Domröse U, Westphal S et al. Cardiac troponin T predicts mortality in patients with end-stage renal disease. Circulation. 2000; 102:1964-1969.
22. Vester B, Rasmussen K. High performance liquid chromatography method for rapid and accurate determination of homocysteine in plasma and serum. Eur J Clin Chem Clin Biochem. 1991; 29:549-554.
23. Porter GA, Norton T, Bennett WB. Troponin T, a predictor of death in chronic haemodialysis patients. Eur Heart J. 1998; 19:N34-N37.
24. Wayand D, Baum H, Schatzle G, Scharf J, Neumeier D. Cardiac troponin T and I in end-stage renal failure. Clin Chem. 2000; 46:1345-1350.
25. De Vriese AS, Verbeke F, Schrijvers BF, Lameire NH. Is folate a promising agent in the prevention and treatment of cardiovascular disease in patients with renal failure? Kidney Int. 2002; 61:1199-1209.
26. House AA, Donnelly JG. Effect of multivitamins on plasma homocysteine and folate levels in patients on hemodialysis. ASAIO Journal. 1999; 45:94-97.
27. Dierkes J, Domröse U, Bosselmann KP, Neumann KH, Luley C. Homocysteine lowering effect of different multivitamin preparations in patientes with end-stage renal disease. J Ren Nutr. 2001; 11:67-72.
28. Bostom AG, Shemin D, Verhoef P et al. Elevated fasting total plasma homocysteine levels and cardiovascular disease outcomes in maintenance dialysis patients. A prospective study. Arterioscler Thromb Vasc Biol. 1997; 11:2554-2558.
29. Robinson K, Gupta A, Dennis V et al. Hyperhomocysteinemia confers an independent increased risk of atherosclerosis in end-stage renal disease and is closely linked to plasma folate and pyridoxine concentrations. Circulation. 1996; 94:2743-2748.
30. Moustapha A, Naso A, Nahlawi M et al. Prospective study of hyperhomocysteinemia as an adverse cardiovascular risk factor in end-stage renal disease. Circulation. 1998; 97:138-141.
31. Homocysteine Lowering Trialists' Collaboration. Lowering blood homocysteine with folic acid based supplements: meta-analysis of randomised trials. BMJ. 1998; 316:894-898.
32. Suwaidi JA, Hamasaki S, Higano ST, Nishimura RA, Holmes DR Jr, Lerman A. Long-term follow-up of patients with mild coronary artery disease and endothelial dysfunction. Circulation. 2000; 101:948-954.

33. Doshi SN, McDowell IF, Moat SJ et al. Folic acid improves endothelial function in coronary artery disease via mechanisms largely independent of homocysteine lowering. Circulation. 2002; 105:22-26.
34. van Guldener C, Lambert J, ter Wee PM, Donker AJ, Stehouwer CD. Carotid artery stiffness in patients with end-stage renal disease: no effect of long-term homocysteine-lowering therapy. Clin Nephrol. 2000; 53:33-41.
35. van Guldener C, Janssen MJ, Lambert J et al. No change in impaired endothelial function after long-term folic acid therapy of hyperhomocysteinaemia in haemodialysis patients. Nephrol Dial Transplant. 1998; 13:106-112.
36. Thambyrajah J, Landray MJ, McGlynn FJ, Jones HJ, Wheeler DC, Townend JN. Does folic acid decrease plasma homocysteine and improve endothelial function in patients with predialysis renal failure? Circulation. 2000; 102:871-875.
37. Williams MJ, Sutherland WH, McCormick MP, de Jong SA, McDonald JR, Walker RJ. Vitamin C improves endothelial dysfunction in renal allograft recipients. Nephrol Dial Transplant. 2000; 16:1251-1255
38. Deicher R, Ziai F, Bieglmayer C, Schillinger M, Horl WH. Low total vitamin C plasma level is a risk factor for cardiovascular morbidity and mortality in hemodialysis patients. J Am Soc Nephrol. 2005; 16:1811-1818.
39. Lim VS, Flanigan MJ. The effect of interdialytic interval on protein metabolism: evidence suggesting dialysis-induced catabolism. Am J Kidney Dis. 1989; 14:96-100.
40. Bergstrom J. Protein catabolic factors in patients on renal replacement therapy. Adv Exp Med Biol. 1989; 260:1-9.
41. Ikizler TA, Wingard RL, Harvell J, Shyr Y, Hakim RM. Association of morbidity with markers of nutrition and inflammation in chronic hemodialysis patients: a prospective study. Kidney Int. 1999; 55:1945-1951.
42. Owen WF Jr, Lew NL, Liu Y, Lowrie EG, Lazarus JM. The urea reduction ratio and serum albumin concentration as predictors of mortality in patients undergoing hemodialysis. N Engl J Med. 1993; 329:1001-1006.
43. Singleton CK, Martin PR. Molecular mechanisms of thiamine utilization. Curr Mol Med. 2001; 1:197-207.
44. Skelton WP 3rd, Skelton NK. Thiamine deficiency neuropathy. It's still common today. Postgrad Med. 1989; 85:301-306.
45. Canner PL, Berge KG, Wenger NK et al. Fifteen year mortality in Coronary Drug Project patients: long-term benefit with niacin. J Am Coll Cardiol. 1986; 8:1245-1255.
46. IONA Study Group. Effect of nicorandil on coronary events in patients with stable angina: the Impact Of Nicorandil in Angina (IONA) randomised trial. Lancet. 2002, 13:1269-1275.
47. Fleischmann EH, Bower JD, Salahudeen AK. Are conventional cardiovascular risk factors predictive of two-year mortality in hemodialysis patients? Clin Nephrol. 2001; 56:221-230.
48. Leavey SF, Strawderman RL, Jones CA, Port FK, Held PJ Simple nutritional indicators as independent predictors of mortality in hemodialysis patients. Am J Kidney Dis. 1998; 31:997-1006.
49. Zager PG, Nikolic J, Brown RH et al. "U" curve association of blood pressure and mortality in hemodialysis patients. Medical Directors of Dialysis Clinic, Inc. Kidney Int. 1998; 54:561-569.

50. Jamison RL, Hartigan P, Gaziano JM et al. Design and statistical issues in the homocysteinemia in kidney and end stage renal disease (HOST) study. Clin Trials 2004; 1:451-460.
51. Heinz J, Dierkes J, Domröse U, Neumann KH, Luley C. Influence of a supplementation with vitamins on morbidity and mortality of ESRD Patients – A multi-center randomly designed double blinded intervention trial; Prismavit – Prospective Intervention Study from Magdeburg with Vitamins. XIV Lipid Meeting Leipzig, Abstract Book 2003.

5

Influence of a Supplementation with Vitamins on Cardiovascular Morbidity and Mortality in Patients with End-stage Renal Disease: Design and Baseline Data of a Randomized Clinical Trial

Based on the Letter to the Editor:

Heinz J, Domröse U, Luley C, Westphal S, Kropf S, Neumann KH, Dierkes J. Influence of a Supplementation with Vitamins on Cardiovascular Morbidity and Mortality in Patients with End-stage Renal Disease: Design and Baseline Data of a Randomized Clinical Trial. Clin Nephrol. 2009; 71:363-365

Hyperhomocysteinemia is present in 80-100% of patients with end-stage renal disease (ESRD). In observational studies, elevated levels of homocysteine were associated with mortality and cardiovascular disease (CVD) in ESRD patients.[1-3] Hyperhomocysteinemia can be reduced by supplementation with folic acid, cobalamin and possibly vitamin B6.[4]

Therefore, in 2002 we started a multi-centre randomized, two-arm, double-blind intervention study in dialysis centres in Germany. The main objective is whether the administration of folic acid, cobalamin and vitamin B6 in high doses will reduce total mortality and the incidence of nonfatal and fatal cardiovascular events. In total, 650 clinically stable patients treated at least for one month with haemodialysis (271 women and 379 men; mean age of 61±13 years) from 33 outpatient units in Northern and Eastern Germany were included. The primary endpoint is total mortality. Secondary endpoints are fatal and nonfatal cardiovascular events (myocardial infarction, instable angina pectoris, heart surgery, stroke, peripheral artery disease). Other secondary endpoints are pulmonary embolism and thrombosis. Shunt thrombosis will be documented but is not regarded as a secondary endpoint.

The sample size calculations with the assumptions of a treatment phase of 3 years, an annual mortality rate of 16%, a reduction of the mortality rate by vitamin supplementation by 30%, an annual drop-out rate of 10%, a two-sided type I error of 5% (adjusted by the O'Brien-Fleming method for one interim analysis) and type II error of 20%, resulted in 350 patients in each treatment group. It became apparent during recruitment that the original number of patients would not be achieved. In an amendment of the original study protocol, the number of patients was reduced to 650 and the follow up period was increased to compensate for the lower number of patients. The actual follow up period therefore is between 2 and 6 years. An interim analysis was made during January 2007 by an external safety committee and revealed that there is no reason to end the trial before schedule. For the final analysis (intention-to-treat) the logrank test, estimation of survival curves with the Kaplan-Meier method and the proportional Cox regression analyses will be used.

Active and 'placebo' vitamin supplements differ only in the content of folic acid, cobalamin and vitamin B6 (Table 1) and are non-distinguishable. It was decided to have a 'vitamin placebo' in order to prevent deficiencies of other vitamins in the placebo group. Influences of the chosen content of folic acid, cobalamin and vitamin B6 in the placebo group on the homocysteine level are not expected. After each haemodialysis treatment (thrice weekly), two

tablets are taken orally under the supervision of the dialysis nurses to ensure compliance. The study protocol was approved by the Ethics Committee of the University Hospital in Magdeburg and the responsible medical associations in the respective federal states of Germany. All procedures were carried out in accordance with the revised declaration of Helsinki. The trial is registered in the Cochrane Renal Group Database, accession number CRG010600027.

Table 1. Study medication: Placebo and active vitamin supplements (dose per tablet)

Component	Placebo supplement	Active supplement
Folic acid	**0.1mg**	**2.5mg**
Vitamin B12 (cobalamin)	**2µg**	**25µg**
Vitamin B6 (pyridoxin)	**0.5mg**	**10mg**
Vitamin B1 (thiamine)	1.2mg	1.2mg
Vitamin B2 (riboflavin)	1.5mg	1.5mg
Niacin	15mg	15mg
Pantothenic acid	6mg	6mg
Biotin	100µg	100µg
Vitamin C (ascorbic acid)	60mg	60mg

Randomized patients showed a high frequency of established risk factors for both cardiovascular disease and mortality. In particular, patients had a high frequency of hypertension (90%), diabetes mellitus (40%) and prevalent cardiovascular disease (48%). LDL-cholesterol was elevated (>4.1mmol/L) in 13% of patients, while 51% of patients had hypertriglyceridemia (>2.3mmol/L). Frequency of current smoking was 17%.

Malnutrition as defined by low body mass index (<23kg/m²) was observed in 26% of patients, low albumin (<40g/L) in 67% of patients and pre-albumin <0.3g/L in 27% of patients. 6% of patients showed three signs of malnutrition, and 27% showed two signs of malnutrition.

Chronic or acute inflammation, as assessed by C-reactive protein levels >5mg/L, was present in 55% of patients. Cardiac troponin T (>0.01ng/mL) was measured in 75% of patients and in 16% a cTnT level >0.1ng/mL, the cut-off indicating acute coronary damage, was observed.

Anaemia (haemoglobin <11g/dL) was prevalent in 34% of patients.

Hyperhomocysteinemia was observed in 94% of the patients. The median [5^{th}–95^{th} percentile] homocysteine level was 29.0 [14.0-63.6] µmol/L. About one-third (30%) of the patients received supplements of water-soluble vitamins on routine basis before study entry.

The study was planned at a time when it was believed that homocysteine lowering with vitamins would also reduce cardiovascular disease. Several trials in patients with cardiovascular disease and intact renal function have, however, resulted in disappointing

results concerning risk reduction.[5,6] Some considerations have to be kept in mind when comparing these trials with the present one. First, these trials involved patients with average homocysteine levels of 12-15µmol/L. ESRD patients often have homocysteine levels which are up to 5-10fold higher. Second, some trials, including the one randomized trial that involved ESRD patients, have been performed in areas with mandatory folic acid fortification of grain products.[7,8] Third, homocysteine-lowering had different effects on different endpoints. Some trials showed a reduction in stroke morbidity, but no effect on cardiovascular mortality or myocardial infarction.[6] Therefore, there is a need for a randomized placebo-controlled intervention study with folic acid, cobalamin and vitamin B6 in patients with ESRD to investigate clinical benefits of such treatment.

References

1. De Vriese AS, Verbeke F, Schrijvers BF, Lameire NH. Is folate a promising agent in the prevention and treatment of cardiovascular disease in patients with renal failure? Kidney Int. 2002; 61:1199-1209.
2. Dierkes J, Domröse U, Westphal S et al. Cardiac troponin T predicts mortality in patients with end-stage renal disease. Circulation. 2000; 102:1964-1969.
3. Bostom AG, Culleton BF. Hyperhomocysteinemia in chronic renal disease. J Am Soc Nephrol. 1999; 10:891-900.
4. Dierkes J, Domröse U, Ambrosch A, Bosselmann HP, Neumann KH, Luley C. Response of hyperhomocysteinemia to folic acid supplementation in patients with end-stage renal disease. Clin Nephrol. 1999; 51:108-115.
5. Bønaa KH, Njølstad I, Ueland PM et al. Homocysteine lowering and cardiovascular events after acute myocardial infarction. N Engl J Med. 2006; 354:1578-1588.
6. Lonn E, Yusuf S, Arnold MJ et al. Homocysteine lowering with folic acid and B vitamins in vascular disease. N Engl J Med. 2006; 354:1567-1577.
7. Jamison RL, Hartigan P, Kaufman JS et al. Effect of homocysteine lowering on mortality and vascular disease in advanced chronic kidney disease and end-stage renal disease: a randomized controlled trial. JAMA. 2007; 298:1163-1170.
8. Spence JD, Bang H, Chambless LE, Stampfer MJ. Vitamin Intervention For Stroke Prevention trial: an efficacy analysis. Stroke. 2005; 36:2404-2409.

Acknowledgments

The authors thank all dialysis patients for participation in this study, all responsible physicians and the staff of dialysis centres for providing assistance with selection of patients, data collection and blood sample collection: Peter Böger-Neuber MD, Claudia Braun MD, Heidrun Deicke MD, Renate Francke MD, Bernhard Friemel MD, Liane Georgiew MD, Klaus Gräfner MD, Michael Hartwig MD, Günter Hofmann MD, Andreas Junghanns MD, Reiner Krainz MD, Ralf Kühn MD, Ilona Lange MD, Andrea Laue MD, Sabine Ludewig MD, Andrea Meier MD, Michael Millington-Herrmann MD, Frank Mönnich MD, Evelyn Nielebock MD, Margitta Oppermann MD, Margit Oppitz MD, Lutz Pannier MD, Karl Heinz Partsch MD, Sylvia Petersen MD, Michael Poley MD, Karlheinz Queck MD, Gerd Rettig MD, Renate Rettkowski MD, Gerhard Riechers MD, Rita Maria Rösch MD, Claudia Rosenburg MD, Silke Röser MD, Karl Sauer MD, Renate Scheel MD, Heike Schlawin MD, Manfred Schlöcker MD, Marianne Schneider MD, Carsten Schröder MD, Reinhard J. Schodrok MD, Michael Schwock MD, Andreas Solf MD, Thomas Steinmetz MD, Carola Striebing MD, Roland E. Winkler MD
Furthermore we thank Christina Grabau and Kerstin Weis for assistance in study medication logistics.

6

B-Vitamins and the Risk for total Mortality and Cardiovascular Disease in End-stage Renal Disease. Results of a Randomized Controlled Trial.

Based on the original article:

Heinz J, Kropf S, Domröse U, Westphal S, Borucki K, Luley C, Neumann KH, Dierkes J. B-Vitamins and the Risk for total Mortality and Cardiovascular Disease in End-stage Renal Disease. Results of a Randomized Controlled Trial. *Submitted*

For reasons of copyright, the original article is only included in abstract-form in the published version of this dissertation. The original article is forthcoming in the journal "Circulation".

Abstract

Background:

In observational studies hyperhomocysteinemia has been found to be a risk factor for total mortality and cardiovascular events in patients with end-stage renal disease. These patients have grossly elevated homocysteine levels that can be lowered by supplementation with folic acid and vitamin B12.

Objective:

We conducted a randomized clinical trial with B-vitamins to reduce homocysteine levels and therefore also cardiovascular events and total mortality.

Design and Setting:

This randomized, double-blind multi-centre study was conducted in 33 dialysis centers in North and East Germany between July 2002 and July 2008.

Patients and Intervention:

650 Patients with end-stage renal disease who were on haemodialysis were assigned at random to two post-dialysis treatments: 5mg folic acid, 50µg vitamin B12 and 20mg vitamin B6 (active treatment) or 0.2mg folic acid, 4µg vitamin B12 and 1.0mg vitamin B6 (placebo) were given three times a week for an average of 2 years.

Main Outcome Measures:

The primary outcome was total mortality and the secondary outcome fatal and non-fatal cardiovascular events.

Results:

The primary outcome occurred in 102 (31%) patients on the active treatment and in 92 (28%) on the placebo (hazard ratio 1.13; 95% confidence interval 0.85 to 1.50, p=0.51). The secondary outcome occurred in 83 (25%) patients on the active treatment and in 98 (30%) on the placebo (hazard ratio 0.80; 95% confidence interval 0.60 to 1.07, p=0.13).

Conclusions:

Increased intake of folic acid, vitamin B12, and vitamin B6 did not reduce total mortality and had no significant effect on the risk of cardiovascular events in patients with end-stage renal disease. (Cochrane Renal Group Prospective Trial Register CRG010600027, www.cochrane-renal.org)

Acknowledgements

We are indebted to all participating dialysis patients, physicians, and dialysis staff. None of these persons received any compensation for their contribution.

7

Overall Discussion and Conclusion

Abbreviations:

RCT	Randomized controlled trial
ESRD	End-stage renal disease
HR	Hazard ratio
PLP	Pyridoxal-5'-phosphate
DOPPS	Dialysis Outcomes and Practice Patterns Study
HOST	Homocysteinemia in Kidney and End Stage Renal disease
VISP	Vitamin Intervention for Stroke Prevention
HOPE-2	Heart Outcomes Prevention Evaluation-2

Since atherosclerotic diseases are the main causes for mortality in the Western industrialized countries,[1,2] research has focused since many years on the identification of treatable risk factors for cardiovascular diseases, such as blood cholesterol or hypertension.

In 1969, the hypothesis was proposed that high homocysteine levels may play a causal role in the development of atherosclerosis, based on the observation of severe atherosclerotic changes in children with homocystinuria.[3] This hypothesis was later modified under the assumption that even moderately elevated homocysteine blood concentrations may be a risk factor for cardiovascular disease. Results of many epidemiological studies, which are summarized in meta-analyses, provided ample support for an association between mild hyperhomocysteinemia and cardiovascular disease, and identified homocysteine as an independent risk factor.[4,5]

The B vitamins folic acid, vitamin B12 and B6 are involved in the metabolism of homocysteine. It was observed, that a supplementation with these vitamins reduces homocysteine plasma levels by approximately 30%.[6] Therefore, it was hypothesized that homocysteine-lowering vitamins may also reduce the risk for cardiovascular events.

Experimental studies with animal models and small trials in humans on the effect of B vitamins on surrogate endpoints, as endothelial function or laboratory endothelial markers,[7,8] have further endorsed the hypothesis of beneficial effects of vitamins and yielded in the initiation of large-scale randomized controlled trials (RCT).

In this context, patients with end-stage renal disease (ESRD) have drawn particular attention because of their high risk for cardiovascular diseases [9,10] and the nearly universal elevation of homocysteine with concentrations up to 150µmol/L.[11] The risk of deficiencies of water-soluble vitamins is higher in this patient group because of the abnormal renal metabolism, inadequate intake, and dialysis losses.[12] In particular, in the context of hyporesponsiveness on recombinant human erythropoietin as a treatment of anaemia in renal failure, vitamin B12 and folic acid deficiencies are reported.[13,14]

The main objective of this thesis was to find a new therapeutic option by supplementation with B vitamins to reduce the high burden of cardiovascular disease and the high mortality in patients with ESRD. In order to investigate the ‘strength of evidence’ for a vitamin supplementation we passed all stages of the hierarchy of research design, from a meta-

analysis of available observational studies to a RCT and aimed to answer the following questions:

Is there an association between homocysteine and cardiovascular disease as well as total mortality in patients with ESRD?

Are plasma levels of homocysteine and related vitamins reduced during the dialysis process and which factors may influence the wash-out?

Does a supplementation with homocysteine lowering vitamins reduce the risk for total mortality and cardiovascular disease in haemodialysis patients?

Main Findings

Whether homocysteine is associated with cardiovascular risk and total mortality in dialysis patients was investigated by a systematic analysis of the available observational studies. Because of the different study designs, separate meta-analyses for retrospective and prospective observational studies were carried out. The main finding of these meta-analyses is a positive association between homocysteine plasma level and risk for cardiovascular disease and mortality in dialysis patients not receiving additional water-soluble vitamins as routine supplementation or folic acid food fortification during the study duration. In these patients, an increase of the plasma homocysteine concentration by 5µmol/L was associated with a significant risk increase of 9% for cardiovascular events (hazard ratio (HR) 1.09; 95% CI, 1.03-1.14; p=0.001) and of 7% for total mortality (HR 1.07; 95% CI, 1.02-1.13; p=0.01). However, in retrospective studies the association between homocysteine and cardiovascular disease was less strong and the pooled results were not significant. We have shown by this meta-analysis that there is a need for a RCT on vitamins, total mortality and cardiovascular disease in patients with ESRD.

In order to examine the reduction of homocysteine and B vitamins during dialysis, plasma levels of homocysteine, folate, cobalamin, pyridoxal-5'-phosphate (PLP), thiamine and riboflavin were measured in 30 patients before and after a haemodialysis session. As in many dialysis centres standard low-flux dialysis membranes are replaced by high-flux membranes, the influence of the type of membrane on the wash-out was investigated. High-flux

membranes have better clearance characteristics for larger molecules, which may also increase the wash-out of water-soluble vitamins. We observed that most of the B vitamins were reduced during the dialysis session. The strongest reduction was observed in plasma levels of folate and PLP. Serum cobalamin was not reduced by the dialysis process. The wash-out of vitamins was independent of the type of dialysis membrane. However, in patients treated with high-flux dialysis the reduction of plasma homocysteine was stronger than in patients treated with standard dialysis. We have shown with this study that water-soluble vitamins are lost through haemodialysis and that a supplementation with B vitamins may be appropriate to avoid vitamin deficiencies as well as to lower homocysteine levels in these patients.

Up to the present, the recommendations for vitamin administration in dialysis patients are based on expert opinions and probable benefits due to the vitamins were not sufficiently established by clinical trials.[12]

In a prospective observational study in a cohort of 102 haemodialysis patients with a follow-up period of 4 years we aimed to investigate whether supplements with multivitamins may have an influence on total mortality. In this study, a strong association between the intake of water-soluble vitamins and reduced mortality was observed (adjusted HR 0.39 [95% CI; 0.19-0.79], $p<0.01$). Because of the observation that multivitamins may possibly affect mortality, a multi-centre RCT was conducted to confirm this association. A total of 650 patients treated with haemodialysis were included in this study and received either high-dose folic acid, vitamin B12, and B6 or a low dose preparation for a period of 2 to 6 years. We investigated the effect of these vitamins on the primary end-point, total mortality, and the secondary endpoint, cardiovascular events. The results of this trial suggest that B vitamin supplements do not reduce total mortality and had no significant influence on the risk of cardiovascular events in patients treated with dialysis (HR 1.13 [95% CI; 0.85-1.50], $p=0.51$ and HR 0.80 [95% CI; 0.60-1.07], $p=0.13$, respectively).

Overall Discussion

Homocysteine as the new risk factor and the possibility of an inexpensive therapy with naturally occurring vitamins have offered a unique target in the prevention of cardiovascular disease. The results of our prospective observational study on multivitamins and total mortality were promising and confirmed by the DOPPS evaluation, which investigated total

mortality in 16,345 dialysis patients from randomly selected renal centres in Europe, Japan and the USA. It was observed that the intake of water-soluble vitamins was associated with a 16% reduction of the relative risk for mortality.[15] However, it is well known that observational studies have certain limitations because of the non-randomized study design. It is possible that the administration of vitamin supplements is a marker for better overall care or a better health and nutritional status. Also socioeconomic factors may have influenced the results. These facts emphasize the need for RCTs to investigate the effects of a therapy and may explain the contradictory results of the observational study and the RCT.

At the time, when our trial was planned, there was a lack of data from RCTs on vitamins and mortality or cardiovascular events. In the meantime, results of other RCTs in patients with chronic kidney disease became available, which are in line with our trial. In the HOST trial, a large-scale trial in patients with advanced kidney disease or end-stage renal disease, no significant effect of the vitamins on total mortality was observed.[16] Also for cardiovascular events our results are comparable to the results of other studies in patients with chronic kidney failure. Most of the studies, including ours, found an insignificant reduction of cardiovascular events by 10 to 20% due to the vitamin supplementation.[16-18]

The therapy effect of B vitamins in subjects without chronic kidney disease has been evaluated in several large-scale RCTs. Up to the present, 7 trials in high risk patients mostly with pre-existing cardiovascular disease have been completed. None of them reported a significant reduction of cardiovascular events or cardiovascular mortality due to the vitamins, although the primary endpoint definition was heterogeneous between studies.[19-25]

So far, these studies are unable to give a clear answer why homocysteine lowering therapy has failed to prevent cardiovascular disease. One of the main reasons may be the relatively low baseline homocysteine levels of the participants. The average homocysteine plasma concentrations in these studies were between 10 and 13µmol/L, which indicates the normal range of plasma homocysteine.[26] Furthermore, some trials have been conducted in areas with mandatory folic acid fortification of grain products. [20,22,23] The fortification policy has been started in 1998 in the USA and Canada.[27,28] The goal of the fortification was to increase folate intake in women of childbearing age and to reduce the risk of neural tube defects. Due to the fortification a profound effect on plasma folate levels and a reduction of mild hyperhomocysteinemia was observed in the population-based Framingham Offspring Study.[29] The eligibility criteria of the VISP trial for the plasma homocysteine concentration had to be reduced from the cut point 10.5µmol/L to 9.5µmol/L for men and 8.5µmol/L for women.[30,31]

A possible attenuation of the effect of folic acid in the respective studies has therefore to be taken into consideration and hence the benefit on cardiovascular events due to reduction of normal homocysteine concentrations is unlikely.

However, also in patients with ESRD, who have much higher homocysteine levels,[32] the therapy with B vitamins failed to reduce cardiovascular risk and mortality.[16-18] Therefore, the question arises whether the "homocysteine hypothesis" can be sustained any longer and whether there is sufficient evidence for a general recommendation of a supplementation with B vitamins.

For patients with ESRD the existing data do not allow a final statement on this question. In all intervention studies in patients with chronic kidney failure, including ours, it was assumed that vitamin therapy may reduce cardiovascular events by 17 to 50%.[16,17] The results of these studies, however, found an event reduction by 10 to 20%, which was not significant. The statistical power for the respective studies did not reach 0.50 to detect an event reduction in this magnitude.[33] For a study power of 0.80, which is taken as the minimum power of a RCT, more than 5000 patients had to be included in the study.

However, we found continuous evidence that the baseline homocysteine levels may have an influence on the development of cardiovascular disease and on the effect of vitamin supplements in patients with ESRD. Already in the meta-analysis of the prospective observational studies we found differences in the results of studies with patients receiving additionally folic acid due to supplementation or food fortification and patients not receiving additional vitamins. An association between homocysteine and cardiovascular disease or total mortality was only found in studies without additional vitamins HR 1.07 [95% CI; 1.02-1.13], p=0.01 and HR 1.09 [1.04-1.15], p=0.001, respectively. It was observed that patients in these studies had average homocysteine levels of about 35µmol/L [11,34-36] and patients with additional vitamin intake had average homocysteine levels of about 25µmol/L,[37-39] which are still values above the reference limit. It may be assumed that only particularly high homocysteine levels, which are regularly found in unsupplemented patients with ESRD and which are comparable to patients with homocystinuria, may cause cardiovascular disease. This hypothesis was subsequently supported by the results of the present RCT. In a subgroup analysis we observed that patients in the highest homocysteine quintile and with homocysteine levels above 41.3µmol/L have profited by the active treatment with B vitamins (HR 0.52 [95% CI; 0.26-1.02], p=0.06 for total mortality and HR 0.48 [95% CI; 0.23-1.01], p=0.05 for cardiovascular events). However, as this is a *post-hoc* analysis, the statistical power was not sufficient to reach final conclusions.

Conclusion and Suggestions for Future Research

At present, based on existing data, including the findings in subjects without renal failure as well as in patients with chronic kidney disease, there is insufficient evidence to justify routine use of homocysteine lowering vitamins for the prevention of cardiovascular disease.

However, on closer examination, it becomes apparent that the studies have severe limitations, which may have decreased the statistical power of the studies and diluted possible preventive effects of the vitamin therapy.

To resolve the relevance of B-vitamins, if any, for the reduction of cardiovascular disease, a meta-analysis on the basis of combined individual participant data from all available RCTs is planned by the B-Vitamin Treatment Trialists' Collaboration.[40] Data should be available on about 32,000 subjects with prior cardiovascular disease in unfortified populations, 14,000 subjects with prior cardiovascular disease in fortified populations, and 6,000 subjects with chronic kidney failure. This meta-analysis should be sufficiently powered to detect a 10% reduction in the cardiovascular event rates.[40,41]

Also, the examination of differentiated end points may contribute to the assessment of the benefit of B-vitamins. For example, the HOPE-2 study reported a significant decrease of strokes by 25% in the vitamin group, a finding that was not discussed in the original publication.[23] In addition, a recent meta-analysis to assess the efficacy of folic acid supplementation on the primary prevention of stroke reported also a significant effect. Folic acid administration reduced the risk of stroke by 18% (RR 0.82; 95% CI [0.68-1.00]; $p = 0.045$), whereas a greater beneficial effect was observed in trials with a longer period of folic acid supplementation (more than 36 months).[42]

Furthermore, we found a strong indication that the homocysteine level before starting a homocysteine-lowering therapy may have a profound effect on the therapy success regarding cardiovascular disease and total mortality in patients with ESRD. It is possible, that only subjects with homocysteine levels exceeding 5 to 10 times the normal range profit from B vitamin supplementation, as it was already observed in patients with homocysteinuria.[43] This hypothesis is supported by *in vitro* studies showing toxic effects of homocysteine in a range between 50 to 1000µmol/L [44,45] and has to be confirmed by sufficiently powered trials in patients with ESRD and particularly high homocysteine levels.

On the other hand, mildly elevated homocysteine levels, rather than being an independent risk factor itself, may constitute a marker for a subliminal renal dysfunction. Results of numerous studies provide ample evidence that homocysteine concentrations increase with decreasing

glomerular filtration rate.[46-48] Furthermore, it was observed that impaired renal function is a very strong predictor of cardiovascular prognosis.[49-52] Thus it cannot be excluded that impaired renal function is the real cause for cardiovascular disease and homocysteine simply reflects the impairment in renal function.

References

1. Cooper R, Cutler J, Desvigne-Nickens P et al. Trends and disparities in coronary heart disease, stroke, and other cardiovascular diseases in the United States: findings of the national conference on cardiovascular disease prevention. Circulation. 2000; 102:3137-3147.
2. Daviglus ML, Lloyd-Jones DM, Pirzada A. Preventing cardiovascular disease in the 21st century: therapeutic and preventive implications of current evidence. Am J Cardiovasc Drugs. 2006; 6:87-101.
3. McCully KS. Vascular pathology of homocysteinemia: implications for the pathogenesis of arteriosclerosis. Am J Pathol. 1969; 56:111-128.
4. Wald DS, Law M, Morris JK. Homocysteine and cardiovascular disease: evidence on causality from a meta-analysis. BMJ. 2002; 325:1202-1206.
5. Homocysteine Studies Collaboration. Homocysteine and risk of ischemic heart disease and stroke: a meta-analysis. JAMA. 2002; 288:2015-2022.
6. Homocysteine Lowering Trialists' Collaboration. Dose-dependent effects of folic acid on blood concentrations of homocysteine: a meta-analysis of the randomized trials. Am J Clin Nutr. 2005; 82:806-812.
7. Zhou J, Møller J, Ritskes-Hoitinga M, Larsen ML, Austin RC, Falk E. Effects of vitamin supplementation and hyperhomocysteinemia on atherosclerosis in apoE-deficient mice. Atherosclerosis. 2003; 168:255-262.
8. Doshi SN, McDowell IF, Moat SJ et al. Folic acid improves endothelial function in coronary artery disease via mechanisms largely independent of homocysteine lowering. Circulation. 2002; 105:22-26.
9. Ikizler TA. Epidemiology of vascular disease in renal failure. Blood Purif. 2002; 20:6-10.
10. Herzog CA, Ma JZ, Collins AJ. Poor long-term survival after acute myocardial infarction among patients on long-term dialysis. N Engl J Med. 1998; 339:799-805.
11. Dierkes J, Domröse U, Westphal S et al. Cardiac troponin T predicts mortality in patients with end-stage renal disease. Circulation. 2000; 102:1964-1969.
12. Fouque D, Vennegoor M, ter Wee P, et al. EBPG guideline on nutrition. Nephrol. Dial. Transplant. 2007; 22:ii45-ii87.
13. Drüeke T. Hyporesponsiveness to recombinant human erythropoietin. Nephrol Dial Transplant. 2001; 7:S25-S28.
14. Schiffl H, Lang SM. Folic acid deficiency modifies the haematopoietic response to recombinant human erythropoietin in maintenance dialysis patients. Nephrol Dial Transplant. 2006; 21:133-137.

15. Fissell RB, Bragg-Gresham JL, Gillespie BW et al. International variation in vitamin prescription and association with mortality in the Dialysis Outcomes and Practice Patterns Study (DOPPS). Am J Kidney Dis. 2004; 44:293-299.
16. Jamison RL, Hartigan P, Kaufman JS et al. Effect of homocysteine lowering on mortality and vascular disease in advanced chronic kidney disease and end-stage renal disease: a randomized controlled trial. JAMA. 2007; 298:1163-1170.
17. Zoungas S, McGrath BP, Branley P et al. Cardiovascular morbidity and mortality in the Atherosclerosis and Folic Acid Supplementation Trial (ASFAST) in chronic renal failure: a multicenter, randomized, controlled trial. J Am Coll Cardiol. 2006; 47:1108-16.
18. Vianna AC, Mocelin AJ, Matsuo T et al. Uremic hyperhomocysteinemia: a randomized trial of folate treatment for the prevention of cardiovascular events. Hemodial Int. 2007; 11:210-216.
19. Baker F, Picton D, Blackwood S, et al. Blinded comparison of folic acid and placebo in patients with ischemic heart disease: an outcome trial. Circulation. 2002; 106:3642 (abstr).
20. Toole JF, Malinow MR, Chambless LE et al. Lowering homocysteine in patients with ischemic stroke to prevent recurrent stroke, myocardial infarction, and death. The Vitamin Intervention for Stroke Prevention (VISP) randomized controlled trial. JAMA. 2004; 291:565-575.
21. Bønaa KH, Njølstad I, Ueland PM et al. Homocysteine lowering and cardiovascular events after acute myocardial infarction. N Engl J Med. 2006; 354:1578-1588.
22. Albert CM, Cook NR, Gaziano JM et al. Effect of folic acid and B vitamins on risk of cardiovascular events and total mortality among women at high risk for cardiovascular disease: a randomized trial. JAMA. 2008; 299:2027-2036.
23. Lonn E, Yusuf S, Arnold MJ et al. Homocysteine lowering with folic acid and B vitamins in vascular disease. N Engl J Med. 2006; 354:1567-1577.
24. Ebbing M, Bleie Ø, Ueland PM et al. Mortality and cardiovascular events in patients treated with homocysteine-lowering B vitamins after coronary angiography: a randomized controlled trial. JAMA. 2008; 300:795-804.
25. Armitage JM, SEARCH Study Collaborative Group. SEARCH (Study of the Effectiveness of Additional Reductions in Cholesterol and Homocysteine): Randomized Comparison of Folic Acid 2 mg Plus Vitamin B12 1 mg Daily versus Placebo for 7 Years in 12,064 Myocardial Infarction Survivors. Circulation. 2008; 118:2310 (abstr)
26. Malinow MR, Bostom AG, Krauss RM. Homocyst(e)ine, diet, and cardiovascular diseases: a statement for healthcare professionals from the Nutrition Committee, American Heart Association. Circulation. 1999; 99:178-182.
27. Food standards: amendment of standards of identity for enriched grain products to require addition of folic acid. Federal Register. 1996; 61:8781-8797.
28. Regulations amending the Food and Drug Regulations (1066). Canada Gazette, Part I. 1997; 131:3702-3737.
29. Jacques PF, Selhub J, Bostom AG, Wilson PW, Rosenberg IH. The effect of folic acid fortification on plasma folate and total homocysteine concentrations. N Engl J Med. 1999; 340:1449-1454.

30. Malinow MR, Chambless LE, Howard VJ, Toole JF. Screening of plasma homocysteine levels in stroke patients for the Vitamin Intervention in Stroke Prevention (VISP) clinical trial. The VISP Investigators. Stroke. 1999; 30:258 (abstr).
31. Bostom AG, Selhub J, Jacques PF, Rosenberg IH. Power Shortage: clinical trials testing the "homocysteine hypothesis" against a background of folic acid-fortified cereal grain flour. Ann Intern Med. 2001; 135:133-137.
32. Billion S 2002 Billion S, Tribout B et al. Hyperhomocysteinaemia, folate and vitamin B12 in unsupplemented haemodialysis patients: effect of oral therapy with folic acid and vitamin B12. Nephrol Dial Transplant. 2002; 17:455-461.
33. Bostom AG, Carpenter MA, Hunsicker L et al. Baseline characteristics of participants in the Folic Acid for Vascular Outcome Reduction in Transplantation (FAVORIT) Trial. Am J Kidney Dis. 2009; 53:121-128.
34. Haraki T, Takegoshi T, Kitoh C et al. Hyperhomocysteinemia, diabetes mellitus, and carotid atherosclerosis independently increase atherosclerotic vascular disease outcome in Japanese patients with end-stage renal disease. Clin Nephrol. 2001; 56:132-139.
35. Ducloux D, Bresson-Vautrin C, Kribs M, Abdelfatah A, Chalopin JM. C-reactive protein and cardiovascular disease in peritoneal dialysis patients. Kidney Int. 2002; 62:1417-1422.
36. Moustapha A, Naso A, Nahlawi M et al. Prospective study of hyperhomocysteinemia as an adverse cardiovascular risk factor in end-stage renal disease. Circulation. 1998; 97:138-141.
37. Bostom AG, Shemin D, Verhoef P et al. Elevated fasting total plasma homocysteine levels and cardiovascular disease outcomes in maintenance dialysis patients. A prospective study. Arterioscler Thromb Vasc Biol. 1997; 17:2554-2558.
38. Sirrs S, Duncan L, Djurdjev O et al. Homocyst(e)ine and vascular access complications in haemodialysis patients: insights into a complex metabolic relationship. Nephrol Dial Transplant. 1999; 14:738-743.
39. Kalantar-Zadeh K, Block G, Humphreys MH, McAllister CJ, Kopple JD. A low, rather than a high, total plasma homocysteine is an indicator of poor outcome in hemodialysis patients. J Am Soc Nephrol. 2004; 15:442-453.
40. Clarke R, Armitage J, Lewington S, Collins R; B-Vitamin Treatment Trialists' Collaboration. Homocysteine-lowering trials for prevention of vascular disease: protocol for a collaborative meta-analysis. Clin Chem Lab Med. 2007;45(12):1575-81.
41. Baigent C, Clarke R. B vitamins for the prevention of vascular disease: insufficient evidence to justify treatment. JAMA. 2007; 298:1212-1214.
42. Wang X, Qin X, Demirtas H et al. Efficacy of folic acid supplementation in stroke prevention: a meta-analysis. Lancet. 2007; 369:1876-1882.
43. Wilcken DE, Wilcken B. The natural history of vascular disease in homocystinuria and the effects of treatment. J Inherit Metab Dis. 1997; 20:295-300.
44. Thambyrajah J, Townend JN. Homocysteine and atherothrombosis--mechanisms for injury. Eur Heart J. 2000; 21:967-974.

45. Ingrosso D, Cimmino A, Perna AF et al. Folate treatment and unbalanced methylation and changes of allelic expression induced by hyperhomocysteinaemia in patients with uraemia. Lancet. 2003; 361:1693-1699.
46. Wollesen F, Brattström L, Refsum H, Ueland PM, Berglund L, Berne C. Plasma total homocysteine and cysteine in relation to glomerular filtration rate in diabetes mellitus. Kidney Int. 1999; 55:1028-1035.
47. Kielstein JT, Salpeter SR, Buckley NS, Cooke JP, Fliser D. Two cardiovascular risk factors in one? Homocysteine and its relation to glomerular filtration rate. A meta-analysis of 41 studies with 27,000 participants. Kidney Blood Press Res 2008; 31:259-267.
48. Veldman BA, Vervoort G, Blom H, Smits P. Reduced plasma total homocysteine concentrations in Type 1 diabetes mellitus is determined by increased renal clearance. Diabet Med. 2005; 22:301-305.
49. Sarnak MJ, Levey AS, Schoolwerth AC, Coresh J, Culleton B, Hamm LL, McCullough PA, Kasiske BL, Kelepouris E, Klag MJ, Parfrey P, Pfeffer M, Raij L, Spinosa DJ, Wilson PW; American Heart Association Councils on Kidney in Cardiovascular Disease, High Blood Pressure Research, Clinical Cardiology, and pidemiology and Prevention. Kidney disease as a risk factor for development of cardiovascular disease: a statement from the American Heart Association Councils on Kidney in Cardiovascular Disease, High Blood Pressure Research, Clinical Cardiology, and Epidemiology and Prevention. Circulation. 2003; 108:2154-2169.
50. Go AS, Chertow GM, Fan D, McCulloch CE, Hsu CY. Chronic kidney disease and the risks of death, cardiovascular events, and hospitalization. N Engl J Med. 2004; 351:1296-1305.
51. Pinkau T, Hilgers KF, Veelken R, Mann JF. How does minor renal dysfunction influence cardiovascular risk and the management of cardiovascular disease? J Am Soc Nephrol. 2004; 15:517-523.
52. Henry RM, Kostense PJ, Bos G et al. Mild renal insufficiency is associated with increased cardiovascular mortality: The Hoorn Study. Kidney Int. 2002; 62:1402-1407.

Summary of the thesis

It has been known for about 40 years, that patients with homocystinuria and extremely high homocysteine blood level develop severe atherosclerotic disease early in their lifetime. This observation led to the hypothesis that moderately increased homocysteine levels may also cause atherogenesis and therefore cardiovascular disease. The association between moderate hyperhomocysteinemia and cardiovascular disease was confirmed by several observational studies in the general population. The interest in this potential risk factor was further augmented by the fact that vitamin B supplements are potentially capable of reducing elevated homocysteine levels. Patients with chronic kidney disease have drawn particular attention since they have a high burden of cardiovascular disease contributing to the high mortality; this however cannot be explained by traditional risk factors, such as elevated blood cholesterol. Identification of treatable risk factors is urgently warranted to improve survival of these patients. Besides the high cardiovascular risk, a nearly universal elevation of blood homocysteine is observed in patients with chronic kidney disease. The present investigations therefore aimed to investigate the effect of vitamin B supplementation on total mortality and cardiovascular disease in patients with end-stage renal disease.

In a systematic analysis of the available observational studies (n=23) homocysteine was identified as a risk factor for total mortality and cardiovascular disease in patients with chronic kidney disease, since they did not receive B vitamins by supplementation or food fortification. An increase of homocysteine level by 5µmol/L was associated with an increased risk for cardiovascular events by 9% (hazard ratio 1.09; 95% CI, 1.03-1.14; p=0.001) and with an increased risk for total mortality by 7% (hazard ratio 1.07; 95% CI, 1.02-1.13; p=0.01).

As homocysteine blood concentration is influenced by B vitamins, we investigated the effect of haemodialysis on blood concentration of water-soluble vitamins in patients with end-stage renal disease (n=30). We found lower plasma concentrations of vitamins with low molecular weight, such as folate and pyridoxal-5'-phosphate, after dialysis. Based on our results we concluded that supplementation with B vitamins on a routine basis may be beneficial to avoid vitamin deficiencies and to lower homocysteine levels in dialysis patients.

In a prospective observational study of a cohort of 102 patients with end-stage renal disease on haemodialysis treatment and with a follow-up period of 4 years we therefore investigated, whether treatment with multivitamins on a routine basis may influence total mortality. Our results showed that dialysis patients receiving multivitamin supplements had a reduction in total mortality (adjusted hazard ratio 0.39; 95% CI, 0.19-0.79; p<0.01).

As a next step this observation had to be confirmed in a randomized controlled trial to justify the evidence for a general recommendation on B vitamins in patients with chronic kidney failure.

In a multi-centre trial, a total of 650 patients treated with haemodialysis were randomly allocated to receive either a high-dose preparation of folic acid, vitamin B6 and vitamin B12 or a low dose preparation of these vitamins for a period of 2 to 6 years. We investigated the effect of these vitamins on the primary endpoint, total mortality, and on the secondary endpoint, cardiovascular events. In this trial high-dose B vitamins had no significant effect on total mortality (hazard ratio 1.13; 95% CI, 0.85-1.50; p=0.51) and cardiovascular events (hazard ratio 0.80; 95% CI, 0.60-1.07; p=0.13) in patients with end-stage renal disease. Results of a *post-hoc* analysis suggest that the initial homocysteine concentration has an influence on the risk reduction for cardiovascular events and total mortality achieved by vitamins. We observed beneficial effects of high-dose vitamins in the patient group with the highest homocysteine level (patients in the highest quintile with homocysteine level higher than 41.3µmol/L). This fits well with the results of the meta-analysis, where homocysteine was associated with cardiovascular disease and total mortality only in patients not receiving vitamin supplements and having therefore highest blood concentration of homocysteine.

The results of the present investigations do not support the hypothesis that a moderately elevated homocysteine level is an independent risk factor in itself in patients with chronic kidney disease. There is no sufficient evidence for a general recommendation about B vitamins in this patient group. On the other hand extremely high homocysteine levels may increase cardiovascular risk as well as total mortality and it may be worthwhile to lower the highest homocysteine levels in order to prevent cardiovascular disease and to improve survival of patients with end-stage renal disease. This assumption needs further confirmation by sufficiently powered trials in patients with particularly high homocysteine levels.

Zusammenfassung

Vor etwa 40 Jahren wurde erstmals beobachtet, dass Patienten mit Homocystinurie und sehr hoher Homocysteinkonzentration im Blut bereits im Kindes- und Jugendalter schwerste atherosklerotische Erkrankungen aufweisen. Daraus wurde in den darauf folgenden Jahren die Hypothese entwickelt, dass auch eine moderate Homocysteinerhöhung ursächlich für atherosklerotische Prozesse und somit ursächlich für kardiovaskuläre Erkrankungen sein kann. Die Assoziation zwischen moderater Hyperhomocysteinämie und kardiovaskulären Erkrankungen wurde durch Ergebnisse zahlreicher Beobachtungsstudien in der Allgemeinbevölkerung bestätigt. Das Interesse an diesem neuen potentiellen Risikofaktor wurde durch die Tatsache verstärkt, dass erhöhte Homocysteinspiegel durch Supplemente mit B-Vitaminen gesenkt werden können. Patienten mit chronischer Nierenerkrankung sind in diesem Zusammenhang von besonderem Interesse, da sie im Vergleich zur Allgemeinbevölkerung ein deutlich erhöhtes kardiovaskuläres Risiko und damit verbunden eine deutlich erhöhte Mortalität aufweisen. Diese Risikoerhöhung lässt sich durch traditionelle Risikofaktoren, wie beispielsweise ein erhöhter Cholesterinspiegel, nicht erklären. Die Identifikation neuer, behandelbarer Risikofaktoren ist somit dringend notwendig, um die Überlebensrate bei diesen Patienten zu verbessern. Neben dem hohen Risiko für kardiovaskuläre Erkrankungen ist bei fast allen Patienten mit chronischen Nierenerkrankungen eine erhöhte Homocysteinkonzentration im Blut zu beobachten. Im Rahmen der vorliegenden Arbeit wurde der Nutzen einer Supplementation mit B-Vitaminen hinsichtlich einer Risikosenkung für Gesamtmortalität und kardiovaskulären Erkrankungen bei Patienten mit terminaler Niereninsuffizienz untersucht.

Durch eine systematische Analyse der bereits in der Literatur vorhandenen Beobachtungsstudien (n=23) konnte zunächst gezeigt werden, dass Homocystein ein Risikofaktor für kardiovaskuläre Erkrankungen und Gesamtmortalität bei Dialysepatienten ist, vorausgesetzt sie nehmen keine zusätzlichen B-Vitamine durch Supplemente oder Lebensmittelanreicherung zu sich. Eine Erhöhung des Homocysteinspiegels um 5µmol/L war mit einem Risikoanstieg um 9% für kardiovaskuläre Erkrankungen (Hazard Ratio 1.09; 95% CI, 1.03-1.14; p=0.001) und um 7% für Mortalität (Hazard Ratio 1.07; 95% CI, 1.02-1.13; p=0.01) assoziiert.

Da die Homocysteinkonzentration im Blut wesentlich von B-Vitaminen beeinflusst wird, wurde außerdem der Effekt der Dialysebehandlung auf die Blutkonzentrationen der wasserlöslichen Vitamine und des Homocysteins bei Patienten mit terminaler Niereninsuffizienz (n=30) untersucht. Insbesondere für Vitamine mit niedrigem

Molekulargewicht, wie Folat und Pyridoxal-5'-Phosphat, wurde eine deutlich niedrigere Plasmakonzentration nach der Dialysebehandlung beobachtet. Aus dieser Untersuchung geht somit hervor, dass eine routinemäßige Supplementation mit B-Vitaminen bei Dialysepatienten angebracht sein kann, um Vitamindefizite zu vermeiden und den Homocysteinspiegel zu senken.

Im Rahmen einer prospektiven Beobachtungsstudie in einer Kohorte von 102 Hämodialysepatienten mit einer Beobachtungszeit von 4 Jahren wurde daher untersucht, ob die routinemäßige Gabe von Multivitaminen das Überleben von Patienten mit terminaler Niereninsuffizienz beeinflussen kann. Hierbei zeigte sich, dass die Vitamingabe mit einer reduzierten Mortalität assoziiert war (adjustierte Hazard Ratio 0.39; 95% CI, 0.19-0.79; $p<0.01$).

Um diese beobachtete Assozation zu bestätigen und die Evidenz für eine allgemeine Empfehlung einer Vitaminsupplementation für Patienten mit chronischem Nierenversagen zu festigen, wurde abschließend eine randomisierte kontrollierte Studie durchgeführt. In die multizentrische Studie wurden insgesamt 650 Hämodialysepatienten in zwei Behandlungsgruppen randomisert. Eine Behandlungsgruppe bekam ein hochdosiertes Präparat mit Folsäure, Vitamin B12 und Vitamin B6 und die andere Behandlungsgruppe bekam ein niedrigdosiertes Präparat für einen Studienzeitraum von 2 bis 6 Jahren. Es wurde der Effekt der Vitamine auf die Endpunkte, Gesamtmortalität und kardiovaskuläre Ereignisse, untersucht. Die Ergebnisse dieser Untersuchung zeigten bei Dialysepatienten keine Reduktion der Gesamtmortalität (Hazard Ratio 1.13; 95% CI, 0.85-1.50; $p=0.51$) und keinen signifikanten Effekt auf kardiovaskuläre Ereignisse (Hazard Ratio 0.80; 95% CI, 0.60-1.07; $p=0.13$) durch hochdosierte B-Vitamine.

Ergebnisse einer *post-hoc* Analyse weisen jedoch darauf hin, dass möglicherweise der Ausgangshomocysteinspiegel den Effekt der Vitamine auf das Risiko für Gesamtmortalität und kardiovaskuläre Erkrankungen beeinflussen kann. Protektive Effekte der Vitamine wurden in der Patientengruppe mit den höchsten Homocysteinkonzentrationen beobachtet (Patienten in der höchsten Quintile mit einem Homocysteinspiegel über 41.3µmol/L). Diese Beobachtung stimmt mit den Ergebnissen der Meta-Analyse überein, wonach Homocystein ausschließlich bei Patienten, die keine Vitaminsupplemente einnahmen und somit höhere Homocysteinkonzentrationen hatten, mit kardiovaskulären Erkrankungen und Gesamtmortalität assoziiert war.

Die Ergebnisse der vorliegenden Untersuchungen unterstützen nicht die Hypothese, dass ein moderat erhöhter Homocysteinspiegel bei Patienten mit chronischen Nierenerkrankungen ein unabhängiger Risikofaktor ist. Die wissenschaftliche Evidenz reicht nicht aus, um eine generelle Empfehlung zur Supplementation mit B-Vitaminen auszusprechen. Andererseits ist eine starke Homocysteinerhöhung mit kardiovaskulären Erkrankungen und Gesamtmortalität assoziert und eine Senkung des Homocysteins in diesen Größenordnungen kann erstrebenswert sein, um möglicherweise kardiovaskuläre Ereignisse zu reduzieren und die Überlebenswahrscheinlichkeit bei Patienten mit terminaler Niereninsuffizienz zu erhöhen. Dieser Zusammenhang sollte in Interventionsstudien mit ausreichender statistischer Power mit Patienten, die einen besonders hohen Homocysteinspiegel haben, überprüft werden.

Annex

Publications

Heinz J, Kropf S, Domröse U, Westphal S, Borucki K, Luley C, Neumann KH, Dierkes J. B-Vitamins and the Risk for total Mortality and Cardiovascular Disease in End-stage Renal Disease. Results of a Randomized Controlled Trial. *submitted*

Heinz J, Kropf S, Luley C, Dierkes J. Homocysteine as a Risk Factor for Cardiovascular Disease in Patients Treated by Dialysis: A Meta-Analysis. Am J Kidney Dis. Apr 2009 [Epub ahead of print]

Heinz J, Domröse U, Luley C, Westphal S, Kropf S, Neumann KH, Dierkes J. Influence of a Supplementation with Vitamins on Cardiovascular Morbidity and Mortality in Patients with End-stage Renal Disease: Design and Baseline Data of a Randomized Clinical Trial. Clin Nephrol. 2009; 71:363-365

Heinz J, Domröse U, Westphal S, Luley C, Neumann KH, Dierkes J. Washout of Water-Soluble Vitamins and of Homocysteine during Haemodialysis - Effect of High-Flux and Low-Flux Dialyzer Membranes. Nephrology 2008; 13:384-389

Domröse U, Heinz J, Westphal S, Luley C, Neumann KH and Dierkes J. Vitamins are associated with survival in patients with end-stage renal disease: a 4-year prospective study. Clinical Nephrology 2007; 4: 221-229

Heinz J, Kästner S, Seewald M, Pötzsch S. Unzureichende Umsetzung der perikonzeptionellen Folsäureeinnahme zur Prävention von Neuralrohrdefekten. Geburtsh Frauenheilk 2006; 66:156-162

Heinz J, Dierkes J. Die Bedeutung von Folsäure für die Gesundheit in verschiedenen Lebensphasen – Teil 1: Folsäure und Neuralrohrdefekte. Ernährung und Medizin 2004; 19(3):141-146

Heinz J, Dierkes J. Die Bedeutung von Folsäure für die Gesundheit in verschiedenen Lebensphasen – Teil 2: Folsäure und kardiovaskuläre Erkrankungen. Ernährung und Medizin 2004; 19(4):194-199

Oral and poster presentations

Heinz J, Kropf S, Luley C, Dierkes J. Homocysteine as a risk factor for mortality and cardiovascular disease in patients treated by dialysis: A meta-analysis. 7th International Conference on Homocysteine Metabolism, Prague (Czech Republic) 2009, Abstract Book p. 42

Heinz J, Kropf S, Luley C, Dierkes J. Ergebnisse der Meta-Analyse „Homocystein – ein Risikofaktor für Gesamtmortalität und kardiovaskuläre Erkrankungen bei Dialysepatienten“ 46. Wissenschaftlicher Kongress der DGE 2009, Proceedings of the German Nutrition Society, Gießen 2009, 13: 76

Heinz J, Kropf S, Luley C, Dierkes J. Ergebnisse der Meta-Analyse: Homocystein – ein Risikofaktor für Gesamtmortalität und kardiovaskuläre Erkrankungen bei Dialysepatienten? 38. Jahrestagung des Arbeitskreises „Klinischer Lipidstoffwechsel“, Maikammer/Pfalz 2008

Heinz J, Kropf S, Luley C, Dierkes J. Ergebnisse einer Meta-Analyse und die Schwierigkeiten auf dem Weg dort hin. Herbstworkshop: Meta-Analysen - Methodik und Anwendungen, Berlin 2008

Heinz J, Dierkes J, Domröse U, Westphal S, Kropf S, Neumann KH, Claus Luley C. Influence of a Supplementation with Vitamins on Cardiovascular Morbidity and Mortality in Patients with End-stage Renal Disease: Design and Baseline Data of a Clinical Trial. XIV International Congress on Nutrition and Metabolism in Renal Disease, Marseille (France) 2008, J Ren Nutr. 2008; 18: S46

Heinz J, Domröse U, Luley C, Neumann KH, Dierkes J: Influence of low and high flux membranes regarding the wash-out of Homocysteine and homocysteine lowering vitamins during hemodialysis; 6th Conference on Homocysteine Metabolism – World congress on Hyperhomocysteinemia, Saarbrücken 2007, Clinical Chemistry and Laboratory Medicine 2007; 45: A84

Heinz J, Domröse U, Neumann KH, Luley C, Dierkes J: Vitaminsupplementation bei Dialysepatienten und ihr Einfluss auf Ernährungsparameter; 44. Wissenschaftlicher Kongress der DGE 2007, Proceedings of the German Nutrition Society, Halle/Saale 2007, 10: 51

Heinz J, Westphal S, Domröse U, Neumann KH, Luley C, Dierkes J: Vitamine und Dialyse – Einfluss von Low- und Highflux-Membranen auf die Auswaschung der Vitamine; 43. Wissenschaftlicher Kongress der DGE, Stuttgart 2006, Proceedings of the German Nutrition Society 2006, 8: 45

Heinz J, Dierkes J, Westphal S, Domröse U, Neumann KH, Luley C: Lipid lowering therapy in ESRD patients in Germany – baseline data from an ongoing prospective study with vitamins; 1st German atherosclerosis symposium, Leipzig 2004, Perfusion 09/2004, 9: 362

Heinz J, Dierkes J, Martens-Lobenhoffer J, Bode-Böger SM, Domröse U, Neumann KH, Luley C: Asymmetric dimethylarginine plasma concentrations in patients with end-stage renal disease and other atherosclerotic risk factors; Vascular NO: From Bench to Bedside, Hannover 2004

Heinz J, Dierkes J, Westphal S, Domröse U, Neumann KH, Luley C: Influence of a supplementation with vitamins on morbidity and mortality of ESRD patients – a multi-center randomly designed double blinded intervention trial; XIV Lipid Meeting Leipzig 2003

Curriculum Vitae

Personal Details

Name:	Judith Heinz
Date of birth:	07.07.1978
Place of birth:	Sangerhausen (Germany)

Research

Since 02/2009	Research assistant; Department of Medical Biometry and Epidemiology, Center for Experimental Medicine, University Medical Center Hamburg-Eppendorf in Hamburg (Germany)
01/2002 - 01/2009	PhD thesis; Institute of Clinical Chemistry and Pathobiochemistry, University Hospital in Magdeburg (Germany) "Hyperhomocysteinemia, Related Vitamins and Mortality in Patients with End-stage Renal Disease: Results of Observational Investigations and a Randomized Controlled Trial."

Education

10/1997 – 12/2001	Study of nutritional sciences at the University of Applied Science in Bernburg (Germany) Diploma thesis: "Reduction of malformations by folic acid - Knowledge and praxis of folic acid intake in women of Saxony-Anhalt"; in the "Monitoring Centre for Malformations in Saxony-Anhalt" in Magdeburg (Germany)
1992 – 1997	Grammar school in Sangerhausen (Germany); A Levels: Biology and English

Professional Training

11/2008	Seminar in advanced management of clinical trials in Leipzig (Germany)
02/2008	Training in Genetic Epidemiology in Lübeck (Germany)
10/2006	Händel-School of Modern Epidemiology "Clinical Epidemiology: An Introduction to Clinical Research", Halle/Saale (Germany)
07/2004	Summer School "Fundamentals of Nutritional Epidemiology" at the German Institute of Human Nutrition in Potsdam-Rehbrücke (Germany)

Erklärung

Hiermit erkläre ich an Eides Statt, dass ich die eingereichte Dissertation **„Hyperhomocysteinemia, Related Vitamins and Mortality in Patients with End-stage Renal Disease: Results of Observational Investigations and a Randomized Controlled Trial.“** selbstständig angefertigt habe und mit dieser wissenschaftlichen Arbeit noch keine vergeblichen Promotionsversuche unternommen wurden. Weiterhin versichere ich, dass ich die zur Erstellung der Dissertationsschrift verwendeten wissenschaftlichen Arbeiten und Hilfsmittel genau und vollständig angegeben habe.

Des Weiteren erkläre ich, dass keine Strafverfahren gegen mich anhängig sind.

_____

Judith Heinz