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The reverse epidemiology of plasma total homocysteine as a mortality risk factor is related to the impact of wasting and inflammation

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Abstract

Background. The reason(s) for the apparently paradoxical 'reverse' association in end-stage renal disease (ESRD) patients in whom a low, rather than a high, total plasma total homocysteine (tHcy) level is an indicator of poor outcome remains unclear. The aim of this study was to examine whether the inverse association maintains, mitigates or reverses after comprehensive multivariate adjustment for the presence of wasting and inflammation as well as other potential confounders.

Methods. We studied 317 ESRD patients starting dialysis therapy. Fasting blood samples were taken for the analyses of tHcy, serum albumin, C-reactive protein (CRP), serum creatinine and plasma folate. Nutritional status was assessed by subjective global assessment (SGA). Survival was followed for up to 66 months; 105 patients died.

Results. Using Kaplan–Meier analysis, a low tHcy concentration ($\leq$30 μmol/l) was associated with higher all-cause and cardiovascular (CV) mortality ($P < 0.05$). Using Cox proportional analysis adjusting for age, gender, glomerular filtration rate = GFR, cardiovascular disease = CVD, plasma folate, total cholesterol and diabetes mellitus, the all-cause and CV mortality still tended to be high for patients with low tHcy. Adding nutritional and inflammation markers (Body mass index = BMI, SGA, serum creatinine, serum albumin and CRP), a low tHcy level was no longer associated with higher mortality but a trend for high tHcy was observed.

Conclusions. The link between wasting inflammation and a low tHcy appears to be responsible for the reverse association between plasma tHcy and clinical outcome in ESRD patients. After adjustment for confounders including nutritional and inflammation markers, a trend towards increased death risk for high, rather than low, tHcy levels was apparent after adjustment.

Keywords: end-stage renal disease; homocysteine; inflammation; mortality; wasting

Introduction

Cardiovascular disease (CVD) is a major cause of the high mortality rate in end-stage renal disease (ESRD) patients. Among several CV risk factors, the markedly increased level of plasma total homocysteine (tHcy) in ESRD patients has been suggested as one independent risk factor for CVD. In the general population, many studies have shown that even mildly elevated plasma tHcy levels are associated with an increased CV [1–3]. However, not all prospective cohort studies are consistent with this finding [2]. The results of large vitamin intervention studies have not been promising, and moderately lowering tHcy levels did not reduce CV events in patients with existing cardiac or vascular disease [4–6]. Relationships between high tHcy levels and CVD as well as worse survival have been reported in ESRD patients [7–10]. Mallamaci *et al.* [8] have recently reported in a cohort of patients with ESRD an association between high tHcy and incident CV events independent of traditional and non-traditional risk factors, such as serum C-reactive protein (CRP) and hypoalbuminaemia. In contrast, recent studies in ESRD patients [11–16] show that a high tHcy concentration is not associated with CVD and in fact is a predictor of improved survival in ESRD.

This unexpected reverse association between tHcy and survival in ESRD patients compared with the general population is not unique for tHcy. Other CV risk factors in the general population, such as cholesterol, blood pressure (BP) and body mass index

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(BMI), have also been shown to exhibit such a reverse association with mortality in dialysis patients [17–19]. The explanations for these reverse associations in dialysis patients are not evident. Several possible reasons have been suggested including survival bias and temporal discrepancies between competitive risk factors [20]. Wasting and inflammation are common in ESRD patients, and have been suggested as causes or at least contributing factors to some of these reverse associations. Indeed, a recent study by Liu *et al*. [21] showed that the presence of inflammation and malnutrition in ESRD alters the association between serum cholesterol and mortality in dialysis patients. Also, Fleischmann *et al*. [22] have suggested that better nutrition might account for the reported risk paradox in haemodialysis (HD) patients in whom overweight and hyperlipidaemia seem to be associated with improved survival.

Recent studies have shown that ESRD patients with signs of wasting and inflammation have a lower plasma tHcy level than patients without wasting and without inflammation, respectively [12,13,15,23,24]. For instance, in a study including 459 HD patients, Ducloux *et al*. [23] showed a trend towards an inverse relationship between tHcy and all-cause and CV mortality in patients who had wasting and inflammation, whereas high tHcy levels independently predict death in patients without wasting and inflammation. However, no association was found between low tHcy levels and poor nutritional status in the Modification of Diet in Renal Disease cohort study [25]. Therefore, the presence of wasting and inflammation may confound the association between tHcy and clinical outcome in ESRD patients. To examine this, we evaluated the association between tHcy level and all-cause as well as CV mortality before and after comprehensive adjustment for several potential confounders including various nutritional and inflammatory markers in ESRD patients.

Materials and methods

Patients

The study protocol was approved by the Ethics Committee of Karolinska Institutet at Karolinska University Hospital Huddinge, Stockholm, Sweden, and informed consent was obtained from each patient. Incident ESRD patients were investigated as part of an ongoing prospective cohort study of atherosclerosis and lipid metabolism [26]. Since originally this patient material was not designed to examine the reverse association between hyperhomocysteinaemia and mortality in ESRD patients, a *post hoc* analysis was done in 317 ESRD patients [195 males and 122 females with a median age 55 (range 22–70) years]. The median glomerular filtration rate (GFR) was 6.4 (range 0.8–16.5) ml/min/1.73 m^2. A total of 244 patients were studied before starting dialysis treatment (median pre-dialysis time 21 days), and 73 patients were studied just after starting dialysis treatment (median post-dialysis time 9 days, see 'Results' section). The study exclusion criteria were age below 20 years or above 70 years, clinical signs of acute infection, acute vasculitis or liver disease at the time of evaluation, or unwillingness to participate in the study. The causes of chronic kidney disease (CKD) were chronic glomerulonephritis in 94 patients (30%), diabetic nephropathy in 103 (32%), polycystic kidney disease in 34 (11%), interstitial nephritis in 14 (4%), nephrosclerosis in nine (3%) and other, or unknown, etiologies in 63 patients (20%). Of all the patients, 118 (37%) had clinical signs of cerebrovascular, CV and/or peripheral vascular disease at the start of the study. Of the 118 patients, 71 had one or more myocardial infarctions or clinical signs of ischaemic heart disease (angina pectoris) or had undergone coronary artery by-pass surgery; 27 patients had peripheral ischaemic vascular disease; 23 had a history of stroke or cerebral bleeding and seven had a history of an aortic aneurysm. A total of 269 patients were on antihypertensive medications. Most of them were on double or triple therapy including angiotensin-converting enzyme inhibitor or angiotensin II receptor antagonists ($n = 181$), calcium channel blockers ($n = 122$) and β-blockers ($n = 170$) with various combinations of these drugs. A total of 57 patients (18%) were on aspirin and 202 (64%) were being treated with erythropoetin. Most patients were treated with other commonly used drugs in ESRD, such as phosphate and potassium binders and diuretics. Patients were routinely supplemented with water-soluble multivitamins (Oralovite®, from Meda AB, Solna, Sweden), which include thiamine, riboflavin, pyridoxine chloride, nicotine amide and ascorbic acid. However, the patients did not receive folic acid or vitamin B12 supplements at the start of the study.

Blood measurements

Fasting blood samples for all investigated laboratory parameters, in each patient, were taken at the same time at the baseline investigation, in appropriate tubes for generation of plasma and serum. The samples were either analysed immediately or stored at −70°C, pending analyses of plasma tHcy, inflammation and nutritional markers as well as other blood analyses in this study. Plasma tHcy was determined with high-performance liquid chromatography, as previously described [27]. Determinations of serum albumin (bromcresol purple), serum CRP (turbidometry), serum creatinine total cholesterol, triglyceride, high-density lipid-cholesterol and haemoglobin were performed by routine procedures in the Department of Clinical Chemistry, Karolinska University Hospital Huddinge. Serum levels of interleukin-(IL-)6 were measured by ELISA kits obtained from Boehringer Mannheim (Mannheim, Germany). Plasma folate was determined with the Dualcount SPNB (solid phase no boil) radioimmunoassay kit from DPC (Diagnostic Product Corporation, Los Angeles, CA, USA). GFR was estimated by the mean of urea and creatinine clearances from a 24 h collection of urine.

Subjective global nutritional assessment

Subjective global nutritional assessment (SGA) was used to evaluate the overall protein-energy nutritional status [28]. SGA includes six subjective assessments, three based on the patient's history of weight loss, incidence of anorexia and incidence of vomiting, and three based on the

physician's grading of muscle wasting, presence of oedema and loss of subcutaneous fat. Based on these assessments, each patient was given a score that reflects the nutritional status as follows: 1 = normal nutritional status, 2 = mild wasting, 3 = moderate wasting and 4 = severe wasting. Patients with an ordinal SGA score between 2 and 4 were grouped together as malnourished.

Outcome ascertainment

Survival was determined from the day of examination, with a median follow-up period of 21.2 (range 0.7–66) months. There was no loss of follow-up of any patient, i.e. all patients could be accounted for in the survival analyses. Each death was reviewed and assigned an underlying cause by an independent physician, who judged the cause of death based on his/her opinion. Autopsies were performed in selected cases. CV mortality was defined as death due to coronary heart disease, sudden death, stroke or complicated peripheral vascular disease.

Statistical analyses

All values are expressed as mean ± SD, unless otherwise indicated. A P-value <0.05 was considered to be statistically significant. Comparisons between two groups were assessed for continuous variables with the Student's unpaired t-test, Mann–Whitney test or chi-square test, as appropriate. Spearman's rank correlation was used to determine correlations between tHcy levels and other variables. To evaluate the sensitivity and specificity of tHcy, as a predictor of mortality, a receiver operating characteristic (ROC) analysis was performed. The optimum cut-off value, with the combination of the highest sensitivity and specificity, was calculated. The more the area under the curve approaches one, the higher the predictive value. The ROC analysis was performed by using the statistical software NCSS 2004 and PASS 2005 (Number Cruncher Statistical Systems, Keysville, Utah, USA). Survival analyses were made with the Kaplan–Meier survival curve or the Cox proportional hazard model. The relative risks for mortality were determined by univariate and multivariate Cox regression analysis and presented as hazard ratio (HR) [95% confidence intervals (CI)]. The Cox proportional-hazards model was used to examine the effects of baseline and follow-up variables on the outcome variables. The goal of the analysis was to assess the HR (analogous to risk ratio or relative risk) of the particular value compared with a reference value (HR = 1). Plots of log [−log (survival rate)] against log (survival time) were performed to establish the validity of the proportionality assumption. Since the proportional-hazards model assumes a constant HR over time, the assumption for each baseline covariate of the Cox proportional-hazards model was tested before the final analysis by evaluating a time-covariate interaction term. The maximum-likelihood test at $P<0.05$ was considered to accept that the HR of the particular variable depended on time. The statistical analysis was performed using statistical software SAS version 9.1 (SAS Campus Drive, Cary, NC, USA 27513).

Result

The median (range) plasma tHcy was 30 (5–239) μmol/l. Of all the patients, 93% had a tHcy level >13.7 μmol/l. This cutoff level is consistent with the 95th percentile of tHcy in healthy subjects [12]. A total of 73 patients in this study had had dialysis therapy for a median of 8 days prior to the study. However, tHcy, CRP, serum albumin, serum creatinine, plasma folate levels, prevalences of inflammation, malnutrition, CVD and diabetes mellitus (DM) did not differ between the 73 patients who had already started dialysis and the rest of the patients in the study (data not shown) and, therefore, all patients were analysed as one group. The characteristics of the patients with tHcy concentration above and below the median level of tHcy are shown in Table 1. The patients with a lower tHcy level had a higher prevalence of wasting, inflammation and DM, compared with the patients with high tHcy levels. Moreover, in the patients with low tHcy, the levels of serum albumin, serum creatinine and HDL were significantly lower, whereas the levels of plasma folate, CRP and IL-6 were significantly higher than in the patients with high tHcy levels. There were no significant differences between the two patient groups regarding gender, BMI, GFR, BP, haemoglobin, cholesterol or triglycerides concentrations.

The plasma tHcy level was negatively correlated with GFR ($\rho=-0.12$, $P<0.05$), plasma folate ($\rho=-0.27$, $P<0.0001$), CRP ($\rho=-0.24$, $P<0.0001$) and IL-6 ($\rho=-0.27$, $P<0.0001$) levels, presence of wasting (SGA>1) ($\rho=-0.23$, $P<0.001$), presence of DM ($\rho=-0.23$, $P<0.0001$) and positively correlated with serum albumin ($\rho=0.45$, $P<0.0001$) and serum creatinine ($\rho=0.24$, $P<0.0001$) levels.

Survival was determined from the day of examination, with a mean follow-up period of 21 (range 1–66) months, with no loss of follow-up of any patient. Within the follow-up period, 105 (33%) patients died of whom 68 (65%) died of CV causes. Table 2 summarizes the characteristics of survivors and non-survivors. The non-survivors had significantly lower concentrations of tHcy ($P<0.001$) compared with the survivors. In accordance, the patients who died due to CVD had a significantly lower concentration of tHcy (35 ± 34 *vs* 39 ± 25 μmol/l, $P<0.01$) compared with the survivors. However, the difference in tHcy levels between patients who died from CV causes and those who died from non-CV causes (35 ± 34 *vs* 29 ± 17 μmol/l, respectively) did not reach significance.

In an attempt to examine whether controlling for risk factors and confounders could alter the results of the survival analysis, we first performed a Kaplan–Meier survival curve, as a reference analysis, and then analysed two adjusted survival curves using a Cox regression model. The cut-off level of tHcy concentration, with the combination of the highest sensitivity and specificity, was 30 μmol/l; this was also almost exactly the same value as the median of tHcy concentration.

Table 1. Baseline characteristic of 317 ESRD and comparison between patients with high plasma tHcy concentrations and patients with low tHcy concentrations

	All	High tHcy (>30 μM)	Low tHcy (≤30 μM)	*P*-value
Number	317	159	158	
Gender, M/F	195/122	97/62	98/60	0.87
Age, year	53 ± 12	51 ± 13	54 ± 12	0.10
BMI, kg/m^2	25 ± 4	25 ± 4	24 ± 4	0.56
GFR, ml/min	7 ± 2	7 ± 2	7 ± 3	0.36
Wasting (SGA>1), %	34	24	43	<0.001
CVD, %	37	33	42	0.11
DM, %	32	24	41	<0.01
tHcy, μmol/l	37 ± 26	52 ± 29	21 ± 6	
tHcy, μmol/l[a]	30 (5–239)	43 (30–239)	22 (5–30)	
Plasma folate, ng/ml	6.2 ± 4.4	5.3 ± 4.4	7.1 ± 10	<0.0001
S-albumin, g/l	33 ± 6	35 ± 5	31 ± 7	<0.0001
S-creatinine, μmol/l	712 ± 250	753 ± 264	670 ± 229	<0.01
Serum CRP, mg/l	18 ± 23	13 ± 12	22 ± 29	<0.001
Serum CRP, mg/l[a]	5 (0.2–218)	3.9 (0.2–119)	7.9 (0. 2–218)	
Interleukin-6, pg/ml	9 ± 10	7 ± 5	11 ± 12	<0.0001
Interleukin-6, pg/ml[a]	6.4 (0.8–112)	5.1 (0.8–46.6)	7.3 (1.1–112)	
Systolic BP, mmHg	151 ± 25	150 ± 26	153 ± 24	0.36
Diastolic BP, mmHg	88 ± 14	88 ± 14	88 ± 13	0.63
Mean BP, mmHg	109 ± 16	108 ± 17	110 ± 15	0.47
Haemoglobin, g/l	105 ± 15	105 ± 15	103 ± 16	0.42
s-Cholesterol, mg/dl	213 ± 58	213 ± 58	209 ± 58	0.70
s-Triglycerides, mg/dl	195 ± 115	186 ± 115	199 ± 115	0.37
HDL-cholesterol, mg/dl	50 ± 23	54 ± 27	46 ± 19	<0.05

[a]Median (range). Significance was set at $P < 0.05$. To convert folate in ng/ml to nmol/l, multiply by 2.266; cholesterol in mg/dl to mmol/l, multiply by 0.02586; triglycerides in mg/dl to mmol/l, multiply by 0.0113.

Table 2. Comparison between survivors and non-survivors ESRD patients

	Survivors	Non-survivors	*P*-value
Number	212	105	
Gender, M/F	126/86	69/36	0.33
Age, year	49 ± 12	60 ± 8	<0.0001
BMI, kg/m^2	24 ± 4	25 ± 5	0.68
GFR, ml/min	6.6 ± 2.3	6.5 ± 2.2	0.72
Wasting (SGA>1), %	21	60	<0.0001
CVD, %	25	63	<0.0001
DM, %	25	47	<0.001
tHcy, μmol/l	39 ± 25	32 ± 29	<0.001
tHcy, μmol/l[a]	32 (5–185)	3.4 (5–239)	
Plasma folate, ng/ml	6.2 ± 4.9	6.6 ± 4.4	0.54
S-albumin, g/l	34 ± 6	30 ± 7	<0.0001
S-creatinine, μmol/l	757 ± 253	620 ± 218	<0.0001
Serum CRP, mg/l	11 ± 20	22 ± 35	<0.001
Serum CRP, mg/l[a]	4 (0.2–134)	11 (0.3–218)	
Interleukin-6, pg/ml	7.3 ± 6.7	12.4 ± 13.3	<0.0001
Interleukin-6, pg/ml[a]	5.1 (0.8–48.4)	8.8 (1.9–112)	
Systolic BP, mmHg	149 ± 24	155 ± 27	0.08
Diastolic BP, mmHg	90 ± 13	85 ± 14	<0.05
Mean BP, mmHg	110 ± 15	108 ± 17	0.62
Haemoglobin, g/l	105 ± 15	103 ± 15	0.38
s-Cholesterol, mg/dl	205 ± 54	220 ± 66	0.08
s-Triglycerides, mg/dl	186 ± 106	195 ± 124	0.77
HDL cholesterol, mg/dl	50 ± 19	50 ± 23	0.87

[a]Median (range). Significance was set at $P < 0.05$. To convert folate in ng/ml to nmol/l, multiply by 2.266; cholesterol in mg/dl to mmol/l, multiply by 0.02586; triglycerides in mg/dl to mmol/l, multiply by 0.0113.

The sensitivity and specificity for this cut-off level were 62 and 61%, respectively. This cut-off level was used for dividing patients into two groups for the comparison of survival rates. In the non-adjusted analysis, low plasma tHcy was associated with high all-cause (HR 1.6; CI 1.1–2.4, $P = 0.01$) and CV (HR 1.8; CI 1.1–2.9, $P = 0.02$) mortality [Figure 1 (A1) and (A2)]. After adjusting for age, gender, GFR, folic acid, total cholesterol, CVD, mean BP and DM (Tables 3 and 4), the high tHcy showed a trend towards inverse relationships with all-cause (HR 1.19; CI 0.62–2.28) and high CV (HR 1.29; CI 0.64–2.57) mortality [Figure 1 (B1) and (B2)]. However, these associations did not reach statistical significance.

In a further analysis [Figure 1 (C1) and (C2)], adjustment for the above variables plus nutritional and inflammation parameters (BMI, SGA, serum creatinine, serum albumin and CRP) (Tables 3 and 4) showed that the previous association between a low tHcy concentration and high mortality rate in Figure 1 (A1, A2) and (B1, B2) disappeared. The HR for low tHcy was now 0.73 (CI 0.33–1.60) for all-cause (C1) and 0.78 (CI 0.34–1.76) for CV mortality (C2). Thus, a high rather than a low tHcy level was now found to be associated with higher mortality, although this difference was not statistically significant (C1 and C2).

Discussion

The findings of this study suggest that the observed inverse association between a high tHcy level and reduced mortality in dialysis patients may be attributed, at least in part, to the influence of various confounders, including markers of wasting and

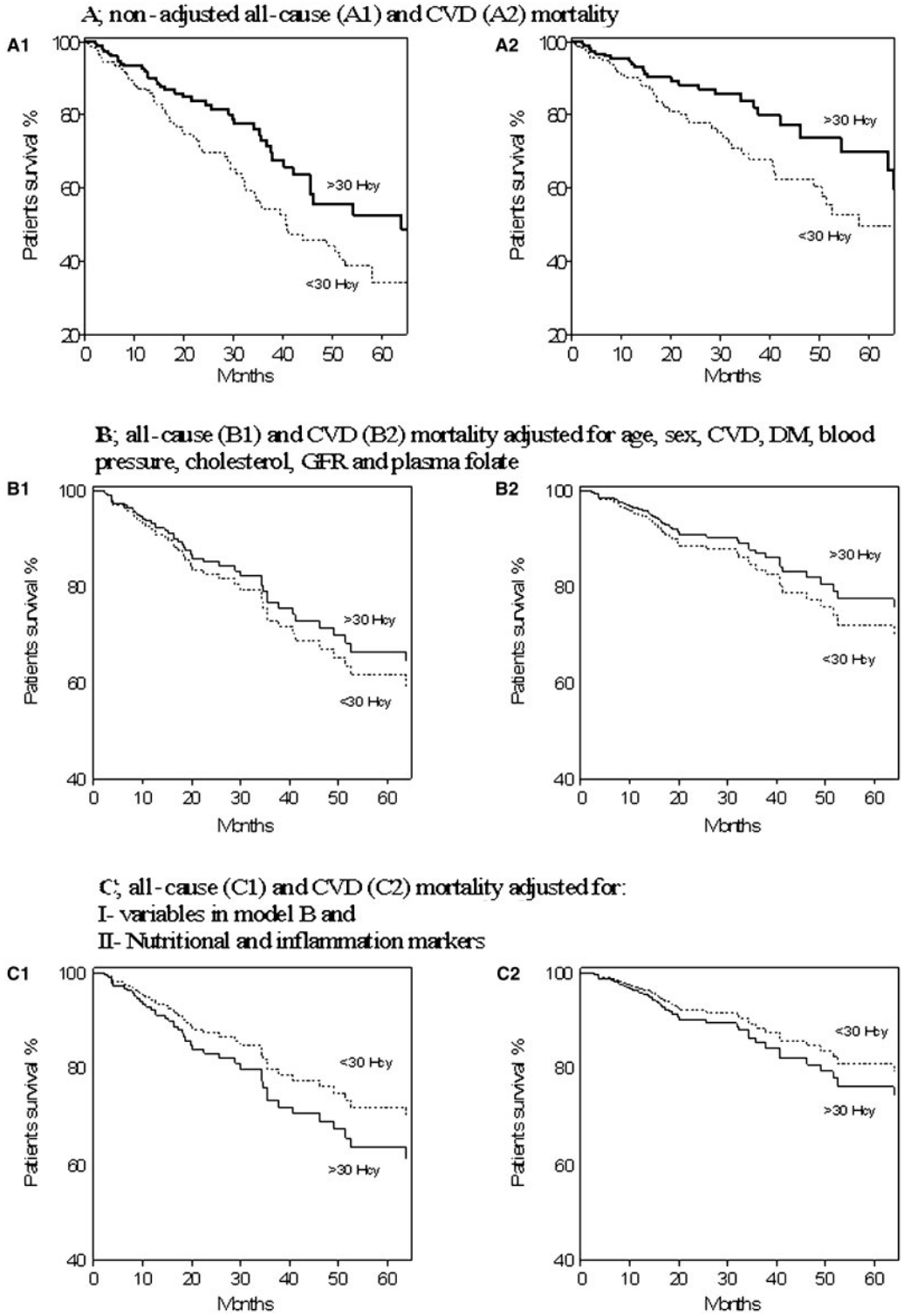

Fig. 1. Probability of survival rate of 317 ESRD patients during 66 months of follow-up with regard to all-cause mortality (left panel) and cardiovascular mortality (right panel) in relation to plasma total homocysteine (tHcy) at the start of dialysis therapy. In an unadjusted analysis (**A**), patients with low tHcy ($\leq$30 µmol/l) concentrations had worse survival than the patients with high tHcy (>30 µmol/l) concentrations. The HR of low tHcy for all cause (**A1**) was 1.6 (CI 1.1–2.4, $P = 0.01$) and for cardiovascular (**A2**) was 1.8 (CI 1.1–2.9, $P = 0.02$). After adjustment for age, gender, GFR, CVD, plasma folate, total cholesterol, mean blood pressure and diabetes mellitus the survival rate for high tHcy was still better, but this difference was not statistically significant (**B**). The HR of low tHcy for all cause mortality (**B1**) was 1.19 (CI 0.62–2.28) and for cardiovascular mortality (**B2**) was1.29 (CI 0.64–2.57). After adjustment for nutritional and inflammation parameters (BMI, SGA, serum creatinine, serum albumin and CRP) plus the variables in (B), (**C**) shows that the 'inverse association', observed in (A) and (B) was no longer present; instead, a high tHcy level tended to be positively associated with high mortality, but this did not reach statistical significance. The HR of low tHcy for all cause mortality (**C1**) was 0.73 (CI 0.33–1.60) and for cardiovascular mortality (**C2**) was 0.78 (CI 0.34–1.76).

inflammation on tHcy levels. In agreement with several previous reports [29], there was a reverse association between a high plasma tHcy level and lower all-cause mortality and CV mortality [Figure 1 (A1) and (A2)] before adjustment for confounding factors. However, following the adjustment for several confounding factors which potentially may influence tHcy concentration, a high tHcy level showed a trend towards a positive association with increased all-cause and CV mortality [Figure 1 (C1) and (C2)]. Although these differences were not statistically significant, the all-cause and CV mortality were, respectively, 27 and 22% higher for high tHcy compared with low tHcy levels.

In contrast to the well-documented association between tHcy and CVD in the general population, the association between tHcy and risk for CVD and mortality is not a consistent finding in ESRD [29]. The fact that a high tHcy level does not show up as a risk factor for CVD in several studies of ESRD patients may appear puzzling. However, the present study as well as several earlier publications [23,29] may partly explain this apparent paradox of 'reverse epidemiology' for tHcy. Recently, Ducloux *et al.* [23] found that tHcy in HD patients with wasting and inflammation was inversely related to all-cause mortality (HR 0.78). However, this association was not inverse in HD patients without wasting and inflammation (HR 1.55) suggesting that a potential interaction between tHcy and wasting-inflammation parameters may have obscured or overwhelmed an independent role for tHcy on survival rate, and that the impact of tHcy is dependent on the wasting and inflammatory status [30].

Table 3. Multivariate Cox proportional hazards for all cause mortality in models 1 and 2

Variable	Model 1				Model 2			
	Hazard ratio	95% CI		*P*-value	Hazard ratio	95% CI		*P*-value
Age, high *vs* low	2.34	1.04	5.27	<0.05	2.46	1.05	5.720	<0.01
Gender, female *vs* male	0.78	0.38	1.61	0.50	0.50	0.20	1.248	0.14
DM, yes *vs* no	2.23	1.33	3.73	<0.01	2.74	1.50	5.00	<0.01
Folic acid, high *vs* low	0.89	0.45	1.70	0.73	1.04	0.51	2.09	0.92
Cholesterol, high *vs* low	1.18	0.97	1.45	0.10	1.16	0.94	1.44	0.16
Mean BP, high *vs* low	1.02	1.01	1.04	<0.05	1.03	1.01	1.06	<0.05
CVD, yes *vs* no	2.15	1.03	4.46	<0.05	2.02	1.13	4.41	<0.01
GFR, ml/min	1.69	1.00	2.84	0.05	2.28	1.22	4.25	<0.01
s-albumin, high *vs* low	–	–	–	–	1.01	0.93	1.08	0.95
SGA, score 1 *vs* score >1	–	–	–	–	0.97	0.42	2.25	0.94
s-Creatinine, high *vs* low	–	–	–	–	0.99	0.99	1.00	0.91
BMI, low *vs* high	–	–	–	–	1.55	0.72	3.37	0.27
Serum CRP, high *vs* low	–	–	–	–	2.36	1.06	5.24	<0.05
tHcy, low *vs* high	1.19	0.62	2.28	0.60	0.73	0.33	1.60	0.43

DM, diabetes mellitus; BP, blood pressure; GFR, glomerular filtration rate; SGA, subjective global assessment; CRP, C-reactive protein; tHcy, plasma total homocysteine; CI, confidence interval.
Age, folic acid, cholesterol, mean BP, s-albumin, s-creatinine, BMI and tHcy were divided into groups by the median values. CRP was divided by the level of ≥10 mg/l. SGA >1 denotes wasting.

Table 4. Multivariate Cox proportional hazard for cardiovascular mortality in models 1 and 2

Variable	Model 1				Model 2			
	Hazard ratio	95% CI		*P*-value	Hazard ratio	95% CI		*P*-value
Age, high *vs* low	1.36	0.66	2.81	0.40	1.32	0.62	2.80	0.47
Gender, female *vs* male	0.71	0.33	1.50	0.37	0.46	0.19	1.14	0.09
DM, yes *vs* no	3.30	1.69	6.45	<0.01	3.24	1.43	7.35	<0.01
Folic acid, high *vs* low	0.70	0.36	1.36	0.29	0.62	0.32	1.21	0.16
Cholesterol, high *vs* low	1.02	0.82	1.26	0.89	1.03	0.82	1.30	0.79
Mean BP, high *vs* low	1.01	0.99	1.03	0.56	1.03	0.98	1.03	0.82
CVD, yes *vs* no	2.52	1.18	5.41	<0.01	2.36	1.07	5.26	<0.01
GFR, ml/min	2.56	1.32	4.99	<0.01	4.66	1.99	10.94	<0.001
s-albumin, high *vs* low	–	–	–	–	0.95	0.89	1.01	0.09
SGA, score 1 *vs* score >1	–	–	–	–	1.24	0.54	2.82	0.61
s-Creatinine, high *vs* low	–	–	–	–	0.99	0.99	1.00	0.94
BMI, low *vs* high	–	–	–	–	1.40	0.63	3.12	0.40
CRP high *vs* low	–	–	–	–	1.25	0.97	2.37	0.21
tHcy, low *vs* high	1.29	0.64	2.57	0.47	0.78	0.34	1.76	0.55

For abbreviations see Table 3.
Age, folic acid, cholesterol, mean BP, s-albumin, s-creatinine, BMI and tHcy were divided into groups by the median values. CRP was divided by the level of ≥10 mg/l. SGA>1 denotes wasting.

Plasma tHcy levels are influenced by many factors, including age and gender. Renal failure and nutritional deficiencies of B-vitamins are the most frequent clinical causes of hyperhomocysteinaemia. In the current study, tHcy concentrations were inversely correlated with plasma folate and GFR. Although tHcy did not correlate with age and gender, the patients with low tHcy were older than the patients with high tHcy levels. Moreover, previous studies [13,24,31] showed lower plasma tHcy levels in ESRD patients with DM. Thus, the high prevalence of DM in ESRD patients may further influence the association between tHcy and outcome in this patient group. This study shows that the mortality rate, after a long-term follow-up of 66 months, was higher in patients with lower tHcy levels in a non-adjusted analysis. We then adjusted for the influence of different factors on tHcy levels, adjusting first for factors known to influence tHcy (age, gender, GFR and plasma folate), as well as for three 'conventional' CVD risk factors (i.e. total cholesterol, mean BP and DM), and the previous history or presence of CVD. After the adjustment for these variables, the difference in survival rate between the two groups was smaller and not significant. However, there was still an 'inverse' relationship and low tHcy had >20% high mortality compared with high tHcy [Figure 1(B1) and (B2)].

Considering that tHcy is a risk factor for CVD, but also linked to various metabolic and nutritional factors that are altered in uraemia, we subsequently tested if inclusion of other potential confounders in the statistical analysis could influence the relationship between tHcy and mortality. Previous studies show that plasma tHcy is lower in dialysis patients with wasting and inflammation as well as in patients with DM [12,13,15,24,31]. Epidemiological studies have shown a strong association between clinical outcomes and both wasting and inflammation in dialysis patients [32–35]. Therefore, it has been suggested that wasting and inflammation might be confounders for the apparent reverse association between tHcy and clinical outcome, especially due to the effects of hypoalbuminaemia, whatever the cause (e.g., malnutrition, catabolic process or inflammation) [13,15,24,36]. Wasting and inflammation decrease levels of serum albumin, which is a major determinant of tHcy levels. In this study, serum albumin was strongly associated with tHcy concentration. Circulating tHcy exists mainly as a protein-bound form with albumin being the main Hcy binding protein and this is reflected by a strong positive correlation between plasma tHcy and serum albumin, as has been reported in ESRD patients [12,13,15]. Another important finding in some of the studies with a reverse association in ESRD patients is the documented strong correlation between tHcy and serum creatinine, even stronger than that seen with serum albumin [13]. Although serum creatinine is metabolically linked to Hcy, the former is also considered as a nutritional index in uraemic patients [37].

Whereas several parameters can be used to assess nutritional status, no single method can be considered to be a gold standard to determine nutritional status in patients with ESRD [38]. Among commonly used parameters, serum albumin is a nutritional marker, but it is also a negative acute-phase reactant influenced by inflammation. Therefore, serum albumin is not a suitable nutritional marker in ESRD patients. Moreover, although SGA has been considered as a reliable method to identify wasting, it may also be a marker of degree of sickness and comorbidity in ESRD patients [38–40]. Hence, the assessment of nutritional status in ESRD patients should include several markers. However, we acknowledge that such an approach may create an 'over-adjustment bias' and, may also decrease the statistical 'power' due to the large number of covariates relative to the number of cases. In this study, tHcy correlated with SGA, serum creatinine, serum albumin and CRP, which are all markers of wasting and inflammation. Previous studies have shown an association between tHcy level and presence of wasting and inflammation [12–15,24].

A recent study by Liu *et al*. [21] showed that the presence of wasting and inflammation in dialysis patients alters the association between cholesterol and mortality in dialysis patients. It is clear from our results that the combination of protein-energy malnutrition and inflammation may account for a substantial amount of the phenomenon of a reversed epidemiological relationship between tHcy and mortality in ESRD patients. This suggests that the vascular patho-physiology is not fundamentally different between ESRD patients and the general population, and that homocysteine is indeed a CV risk factor also in ESRD patients, considering that hyperhomocysteinaemia is highly prevalent in uraemic patients. It should be noted that the reduced form of Hcy, rather than tHcy, may be the Hcy fraction that exerts the toxic effects on blood vessels. However, to the best of our knowledge, there are no studies that have evaluated the putative association between the levels of the reduced form of Hcy and CV events.

Our study may have a number of limitations: first, the limited sample size could have an impact on the statistical power. Indeed, our multivariate adjusted results could have reached statistical significance if we had a larger sample size. Therefore, more studies including larger numbers of patients are required. Second, the results of this analysis may not necessarily be conclusive for the entire ESRD patient population, because non-dialysed patients (including those with a kidney transplant) are not represented here. Also, due to exclusion of patients >70 years of age the study subjects were younger than typical ESRD patient populations. Finally, a survival bias due to differences in the observation time and the actual survival at the start of the study may contribute to improved survival at this stage of CKD. However, our study is one of the first ones [23] that seek to explore the true aetiology of the reverse epidemiology of tHcy in dialysis patients. Nonetheless, such studies

do not provide evidence for causality, and randomized control trials are therefore required to explain such associations.

In summary, the inclusion of several confounders known to be associated with tHcy (age, gender, GFR and plasma folate) or well-established risk factors in the general population (serum cholesterol, mean arterial BP, CVD and DM) did not alter the presumably flawed or 'reverse' relationship between tHcy and outcome in incident ESRD patients starting renal replacement therapy. However, following the adjustment for the presence of wasting and inflammation a high tHcy was associated with higher mortality than a low tHcy in this group of ESRD patients. Thus, these results suggest that wasting and inflammation, beside the other confounders, share the responsibility for the reverse association between tHcy and clinical outcome in ESRD patients. As the relationship between tHcy and nutrition/inflammation status is an important confounding factor in the evaluation of hyperhomocysteinaemia as a risk factor in ESRD patients, the present findings therefore strongly support that the influence of wasting and inflammation on tHcy levels should be taken into consideration when evaluating tHcy as a risk factor for morbidity and mortality in ESRD patients.

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