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Serum homocysteine level and protein intake are related to risk of microalbuminuria: The Hoorn Study[1]

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Serum homocysteine level and protein intake are related to risk of microalbuminuria: The Hoorn Study.

Background. Microalbuminuria (MA) is a strong predictor of cardiovascular disease, but its causes are incompletely understood. Hyperhomocysteinemia is a recently recognized risk factor for cardiovascular disease independent of established risk factors. It is not known whether hyperhomocysteinemia is associated with MA, and thus could be a possible cause of microalbuminuria.

Methods. We studied an age-, sex- and glucose-tolerance-stratified random sample of a 50- to 75-year-old general Caucasian population (N = 680). The urinary albumin-to-creatinine ratio (ACR) was measured in an early morning spot urine sample. MA was defined as an ACR $>$ 3.0 mg/mmol.

Results. The prevalence of MA was 4.3% (13 of 304) in subjects with normal glucose tolerance, 9.2% (17 of 185) in impaired glucose tolerance and 18.3% (30 of 164) in non-insulin-dependent diabetes mellitus (NIDDM); it was 3.7% (15 of 402) in subjects without hypertension and 17.9% (45 of 251) in those with hypertension. After adjusting for age, sex, glucose tolerance category, hypertension, dyslipidemia and smoking, the odds ratio [OR; 95% confidence interval (95%CI)] for MA per 5 μmol/liter total homocysteine increment was 1.33 (1.08 to 1.63). Additional adjustment for HbA_{1c}, waist-hip ratio, protein intake and serum creatinine did not attenuate the association between MA and total homocysteine. A 0.1 g/kg · day increment of protein intake was also associated with an increased risk for MA after adjustment for age, sex, classical risk factors and serum total homocysteine [OR (95% CI); 1.20 (1.08 to 1.32)].

Conclusion. Both hyperhomocysteinemia and protein intake are related to microalbuminuria independent of NIDDM and hypertension. Hyperhomocysteinemia may partly explain the link between MA and increased risk of cardiovascular disease.

A slightly elevated urinary albumin excretion rate, so-called microalbuminuria, is a strong predictor of cardiovascular morbidity and mortality in both non-insulin-dependent diabetes mellitus (NIDDM) and non-diabetic individuals [1, 2]. The increased risk of cardiovascular disease in individuals with microalbuminuria is only partly due to a higher prevalence of established risk factors such as diabetes, hypertension, smoking and dyslipidemia. The pathophysiological basis of the association between microalbuminuria and cardiovascular disease remains to be elucidated. The most widely held view is that microalbuminuria indicates underlying generalized vascular, possibly endothelial, dysfunction [3, 4]. Therefore, microalbuminuria and atherothrombotic disease may have certain pathogenetic mechanisms in common.

Hyperhomocysteinemia is a recently recognized risk factor for cardiovascular disease independent of established risk factors [5, 6]. It is not known whether hyperhomocysteinemia is associated with and thus a possible cause of microalbuminuria. Therefore, we investigated this issue in a 50- to 75 year-old general population, an age range in which both hyperhomocysteinemia [7, 8] and microalbuminuria [2] are known to be relatively common.

A high protein intake may increase the risk of developing microalbuminuria [9, 10] and conceivably may also increase serum total homocysteine (tHcy) levels [11]. It is thus a potential confounder of the relationship, if any, between hyperhomocysteinemia and microalbuminuria. We therefore specifically examined the relationships between protein intake, serum total homocysteine, and presence of microalbuminuria.

METHODS

The Hoorn Study is a cross-sectional survey of glucose tolerance and other cardiovascular risk factors in a 50- to 75-year-old general Caucasian population conducted from 1989 to 1992. A random sample of all men and women aged 50 to 75 years was drawn from the municipal population registry office of Hoorn (the Netherlands); 2,484 subjects participated (response rate 71%). The presence of microalbuminuria was investigated (as detailed below) in an

[1] **See *Editorial* by Robinson and Dennis, p 281**

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age-, sex- and glucose-tolerance-stratified random subsample ($N = 680$, response rate 96%), which was constructed by means of a second sampling, as described previously in detail [12]. The Hoorn Study was approved by the Ethical Review Committee of the University Hospital Vrije Universiteit Amsterdam. Informed consent was obtained from all participants.

An early morning first voided spot urine sample was collected. Urinary albumin was measured by rate nephelometry (Array Protein System; Beckman, Ireland) with a threshold of 6.2 mg/liter and intra- and inter-assay coefficients of variation of $\leq 5\%$ and $\leq 8\%$, respectively. Urinary creatinine was measured by a modified Jaffé method. The urinary albumin-to-creatinine ratio (ACR) was calculated. For those urine samples with an albumin concentration less than the threshold, the value 6.2 was taken to calculate the ACR. An ACR ≤ 3.0 mg/mmol was defined as normoalbuminuric, an ACR >3.0 mg/mmol as (micro)albuminuric and an ACR >30.0 mg/mmol as macroalbuminuric. [An ACR of 3 to 30 mg/mmol is approximately equivalent to an albumin excretion rate (AER) of 30 to 300 mg/24 hours] [13, 14]. We also considered a lower cut-off value (ACR $>$ 2.0 mg/mmol) to define (micro)albuminuria. To investigate the reproducibility of the ACR, we collected a second urine sample from a representative sample of 185 subjects in similar fashion. Of all subjects ($N = 680$), 24 urine samples were missing. In addition, we excluded three subjects from further analyses because no serum was available for homocysteine measurement. Thus, analyses were performed on 653 subjects, of whom 185 had an ACR on the basis of the mean of two measurements. By utilizing the available duplicate measurements we reduced the effect of possible misclassification of (micro)albuminuric subjects. Finally, the presence of leukocytes was evaluated by microscopy and scored as positive (>5 leukocytes per high-power field) or negative; a microscopic report was missing in 60 of the available urine samples.

Measurement of serum total homocysteine

Fasting blood samples were centrifuged within one hour following collection. Serum was stored at -20°C for six years. There is good evidence that serum tHcy levels are stable for 10 years or more [15]. Serum total (free plus protein bound) homocysteine level was measured by using tri-n-butylphosphine as the reducing agent and ammonium 7-fluorobenzo-2-oxa-1,3-diazole-4-sulphonate as the thiol-specific fluorochromophore, followed by HPLC with fluorescence detection [16]. The intra- and interassay coefficients are 2.1% and 5.1%.

Other procedures

We measured fasting serum total cholesterol, high density lipoprotein (HDL)-cholesterol and triglycerides by enzymatic techniques (Boehringer-Mannheim, Mannheim, Germany). The Friedewald formula was used to calculate the low density lipoprotein (LDL) cholesterol concentration, except in subjects with serum triglyceride levels >8.0 mmol/liter ($N = 3$) [17]. Serum creatinine was measured by the modified Jaffé method. The creatinine clearance was calculated from serum creatinine, using the Cockcroft and Gault formula [18]. Normal renal function and mild and moderate renal failure were defined as creatinine clearance >80, 51 to 80 and 24 to 50 ml/min, respectively. (There were no subjects with creatinine clearance <24 ml/min.) Immunospecific insulin was measured in serum by a double-antibody radioimmunoassay (lot SP21; Linco Research, St. Louis, MO, USA). The inter-assay coefficient of variation was 6%. The lower limit of sensitivity was 12 pmol/liter. Hypercholesterolemia was defined as total cholesterol ≥ 6.5 mmol/liter and/or the current use of cholesterol-lowering medication. Dyslipidemia was defined as levels of HDL-cholesterol in the lowest (≤ 1.02 mmol/liter) and/or levels of triglycerides in the highest quartile (≥ 2.10 mmol/liter). Hypertension was defined as a blood pressure ≥ 160 mm Hg systolic and/or ≥ 95 mm Hg diastolic and/or the current use of antihypertensive medication. Impaired glucose tolerance (IGT) and NIDDM were defined according to the WHO criteria (1985) [19]. Subjects were classified as either nonsmokers or current smokers. The waist-hip ratio was measured as previously described [12]. Body mass index was calculated as weight divided by height squared (kg/m^2). Information on protein (animal and vegetable) intake was obtained by a self-administered validated semi-quantitative food frequency questionnaire ($N = 638$) [20]. All laboratory measurements were carried out by technicians unaware of the subjects' history of cardiovascular disease and glucose tolerance status.

Statistical analysis

Variables are presented as mean $\pm$ SD, number (percentage of the total) or, in case of skewed distribution, median and interquartile range (IQR) or geometric mean. Differences between subjects with normo- and those with (micro)albuminuria were tested with Student's t or Wilcoxon's rank sum test for continuous variables and Pearson's chi-square test for frequency measures. Associations of cardiovascular risk factors with serum tHcy level (logarithmically transformed) were studied by calculating Pearson's correlation coefficients. The kappa coefficient was calculated to estimate the agreement of the ACR >3.0 mg/mmol between two measurements.

We performed logistic regression analyses to study the association of serum tHcy with (micro)albuminuria. We calculated odds ratios (ORs) and 95% confidence intervals (CIs) per 5 μmol/liter (about 1 SD) increment of serum tHcy, assuming a linear logistic relationship between homocysteine and risk of (micro)albuminuria. We used multiple logistic regression analysis to control for age, sex, glucose tolerance category, hypertension, current smoking and dyslipidemia. To control for confounding as thoroughly

Table 1. Characteristics of the subjects

	Normoalbuminuria ACR ≤ 3.0 mg/mmol $N = 593$	(Micro)albuminuria ACR > 3.0 mg/mmol $N = 60$	P value[a]
Men *%*	48.1	45.0	0.7
Age *years*	63.9 (7.1)	67.3 (6.7)	< 0.0001
Body mass index kg/m^2	27.1 (3.7)	28.2 (6.0)	0.2
Waist-hip ratio	0.91 (0.09)	0.94 (0.07)	0.003
Blood pressure *mm Hg*			
Systolic	138 (19)	154 (21)	< 0.0001
Diastolic	82 (10)	87 (13)	< 0.0001
Hypertension *%*	34.7	75.0	< 0.0001
Use of antihypertensives *%*			
Angiotensin-converting enzyme inhibitor	4.7	10.0	0.09
Other	19.7	41.7	< 0.0001
Impaired glucose tolerance *%*	28.3	28.3	1.0
Non-insulin-dependent diabetes mellitus *%*	22.6	50.0	< 0.0001
Current smoker *%*	28.9	25.4	0.6
Protein intake *g/kg · day*			
total	0.97 (0.27)	1.04 (0.39)	0.2
animal	0.68 (0.22)	0.73 (0.29)	0.1
vegetable	0.30 (0.11)	0.31 (0.17)	0.3
HbA_{1c} *% of hemoglobin*	5.8 (1.2)	6.5 (1.7)	0.002
Fasting insulin *pmol/liter*	84 (63–114)	90 (70–143)	0.007
Total cholesterol *mmol/liter*	6.7 (1.2)	6.5 (1.2)	0.3
LDL cholesterol *mmol/liter*	4.6 (1.1)	4.3 (1.0)	0.06
HDL cholesterol *mmol/liter*	1.3 (0.4)	1.2 (0.3)	0.1
Triglycerides *mmol/liter*	1.5 (1.1–2.1)	1.5 (1.1–2.8)	0.1
Dyslipidemia *%*	38.0	48.3	0.1
Creatinine *μmol/liter*	91 (16)	104 (35)	0.005
Creatinine clearance[b] *ml/min*	75 (18)	67 (20)	0.001
Mild renal failure[c] *%*	58.7	56.7	0.8
Moderate renal failure[d] *%*	5.6	18.3	< 0.0001
Total homocysteine *μmol/liter*	11.4 (9.4–14.2)	12.7 (10.0–15.5)	0.05

Data are presented as mean (standard deviation), percentage of the total or median (interquartile range). ACR is the albumin-to-creatinine ratio. Dyslipidemia was defined as levels of HDL-cholesterol in the lowest (≤1.02 mmol/liter) and/or levels of triglycerides in the highest quartile (≥2.10 mmol/liter).

[a] Tested with Student's *t*-test or Wilcoxon's rank sum test for continuous variables and Pearson's chi-square test for frequencies

[b] Calculated from serum creatinine using the Cockcroft and Gault formula

[c] Creatinine clearance 51 to 80 ml/min

[d] Creatinine clearance 24 to 50 ml/min

as possible, we used, in all analyses, four categories of glucose tolerance: normal glucose tolerance (NGT), IGT, newly detected and known NIDDM. We also tested models that in addition included HbA_{1c}, waist-hip ratio (or BMI), protein intake (g/kg · day), serum creatinine or creatinine clearance and/or fasting insulin level. To evaluate a possible modifying role of other risk factors, we repeated the previous analyses in strata of sex, diabetes, hypertension, dyslipidemia, hypercholesterolemia and current smoking. In addition, we investigated product terms in the logistic regression models. We also evaluated a possible dose-response relation by calculating ORs for (micro)albuminuria for several ranges of homocysteine concentrations with values of serum tHcy equal to or less than 10 μmol/liter as the reference category. By using this procedure we evaded assumptions about linearity.

Finally, the associations between animal and vegetable protein intake and (micro)albuminuria were investigated. We evaluated a possible modifying role of hypertension and glycemic control (HbA_{1c}). A possible dose-response relation was investigated by calculating ORs for (micro)albuminuria for several ranges of daily protein intake with values of total protein intake equal to or less than 0.75 g/kg · day as the reference category. All reported P values are two-tailed. All analyses were performed with SPSS for Windows 7.5.2.

RESULTS

The prevalence of (micro)albuminuria was 9.2% (60 of 653). The kappa coefficient (95% CI) of two urine sample measurements was 0.53 (0.28 to 0.77) for ACR >3.0 mg/mmol, indicating a moderate agreement. As compared to subjects with normoalbuminuria, those with (micro)albuminuria were older, had a higher waist-hip ratio and systolic and diastolic blood pressures, more often had NIDDM and moderate renal failure, and had higher serum levels of creatinine and tHcy (Table 1). The prevalence of (micro)albuminuria was 4.3% (13 of 304) in subjects with NGT, 9.2% (17 of 185) in IGT and 18.3% (30 of 164) in NIDDM; it was 3.7% (15 of 402) in subjects without

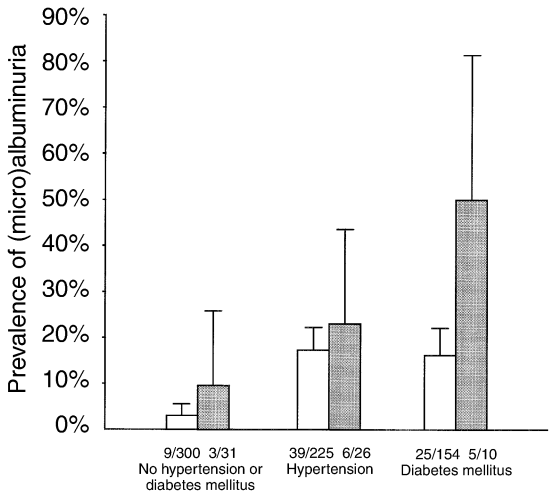

Fig. 1. Prevalence of (micro)albuminuria according to absence or presence of hyperhomocysteinemia (>18.0 μmol/liter) in subjects without or with hypertension and/or diabetes. Symbols are: (□) homocysteine levels ≤ 18 μmol/liter; (▨) homocysteine levels > 18 μmol/liter. Risk of (micro)albuminuria increases markedly above this cut-off value (see Fig. 2). The error bars represent the upper half of the 95% confidence intervals. Number of subjects with (micro)albuminuria of each subgroup are presented below the bars.

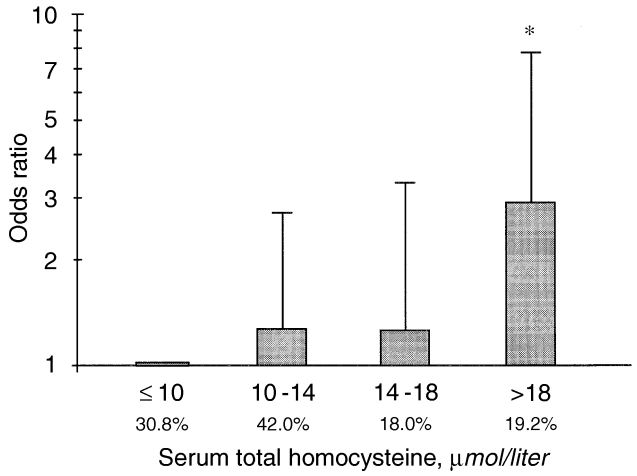

Fig. 2. Odds ratio for (micro)albuminuria according to serum total homocysteine level adjusted for age, sex, impaired glucose tolerance/diabetes mellitus, hypertension and protein intake. The reference category was serum total homocysteine values equal to or less than 10 μmol/liter. The percentages of the population under study for each serum total homocysteine range are presented. Error bars represent the upper half of the 95% confidence intervals. *$P < 0.05$, significantly different from the reference category. P for trend = 0.06. (Note: A logarithmic scale was used since the odds ratio is a multiplicative measure of association, that is, equal differences on the logarithmic scale correspond to equal ratios between the odds ratios.)

hypertension and 17.9% (45 of 251) in those with hypertension. Figure 1 shows the prevalence of (micro)albuminuria according to the absence or presence of hyperhomocysteinemia (>18 μmol/liter) in subjects without or with hypertension and/or diabetes mellitus. We chose this cut-off value since risk of (micro)albuminuria increased markedly above this value (Fig. 2).

The median serum tHcy level was 12.2 μmol/liter (IQR, 10.0 to 15.4) in men and 11.0 μmol/liter (IQR, 9.1 to 13.6) in women. Serum tHcy levels correlated with age ($r = 0.20$; $P < 0.0001$), serum creatinine ($r = 0.41$; $P < 0.0001$), systolic blood pressure ($r = 0.11$; $P = 0.004$), waist-hip ratio ($r = 0.10$; $P = 0.008$), fasting insulin ($r = 0.08$; $P < 0.05$), and inversely with creatinine clearance ($r = -0.3$; $P = 0.01$) and animal protein intake ($r = -0.16$; $P < 0.0001$), but not with vegetable protein intake ($r = -0.1$, $P = 0.1$), BMI ($r = -0.05$; $P = 0.3$), diastolic blood pressure ($r = 0.04$; $P = 0.3$), serum total cholesterol ($r = 0.01$; $P = 0.8$), HDL cholesterol ($r = -0.07$; $P = 0.06$) or HbA_{1c} ($r = -0.02$; $P = 0.6$). The geometric mean serum tHcy level in subjects with moderate and mild renal failure and those with normal renal function were 14.7, 12.4 and 10.6 μmol/liter (P for trend < 0.0001).

A 5 μmol/liter increment of serum tHcy was associated with an increased risk of (micro)albuminuria independent of classical risk factors (Table 2, model 1; Fig. 1). Additional adjustment for serum creatinine (Table 2, model 2) or creatinine clearance did not materially change the ORs, nor did inclusion of total cholesterol, triglycerides, HDL and LDL cholesterol, fasting insulin, systolic blood pressure and/or BMI in the model (data not shown). Exclusion of subjects ($N = 7$) who had an ACR >3.0 mg/mmol based on an albumin concentration of 6.2 mg/liter (= threshold) yielded similar results (data not shown). If (micro)albuminuria was defined as an ACR >2.0 mg/mmol, the ORs (95% CIs) for models 1 and 2 were 1.27 (1.04 to 1.54) and 1.25 (1.02 to 1.55).

Macroalbuminuria was present in seven subjects. After adjustment for age and sex, the OR (95% CI) for macroalbuminuria per 5 μmol/liter increment serum tHcy was 1.33 (1.02 to 1.72). After exclusion of the seven macroalbuminuric subjects and adjustment for age, sex and classical risk factors, the OR for microalbuminuria (ACR: 3−30 mg/mmol) per 5 μmol/liter increment serum tHcy was 1.28 (1.01 to 1.61). Since the strength of the association between micro- or macroalbuminuria and serum tHcy did not differ substantially, we pooled all subjects with an ACR >3.0 mg/mmol.

Angiotensin-converting enzyme inhibitors and other antihypertensives can reduce the urinary albumin excretion. The geometric means of tHcy of subjects who did not or did use an angiotensin-converting enzyme inhibitor were 11.8 and 12.5 μmol/liter, respectively ($P = 0.5$). Adjustment for use of angiotensin-converting enzyme inhibitors (yes/no) in addition to classical risk factors (model 1 in Table 2) did not materially change the association between tHcy and (micro)albuminuria [OR (95% CI) 1.32 (1.07 to 1.62)]. Adjustment for use of antihypertensives (yes/no) yielded similar results. The geometric means of tHcy of subjects without or with leukocyturia were 11.9 and 12.4 μmol/liter,

Table 2. Odds ratios (ORs; 95% confidence intervals) for (micro)albuminuria per 5 μmol/liter increment of serum total homocysteine

(Micro)albuminuria ACR mg/mmol	Cases	Crude	Age- & sex-adjusted	Model 1	Model 2
		OR			
>3.0	60	1.30 (1.09–1.54)	1.30 (1.08–1.56)	1.33 (1.08–1.63)	1.28 (1.03–1.59)

Model 1 was adjusted for age, sex, glucose tolerance category, hypertension (yes/no), current smoking (yes/no) and dyslipidemia (yes/no). Model 2 was model 1 + additional adjustment for protein intake and serum creatinine. Abbreviation ACR is albumin-to-creatinine ratio.

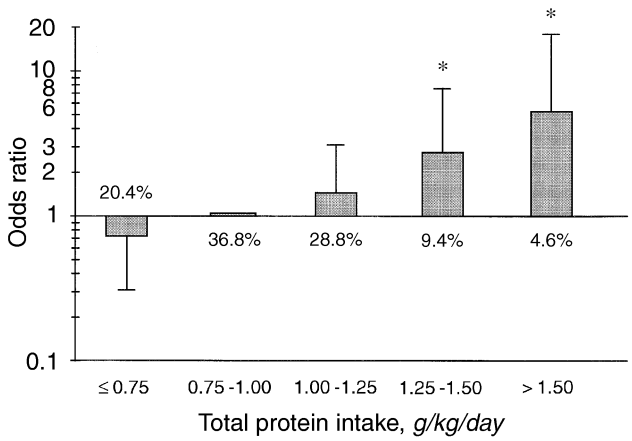

Fig. 3. Odds ratio for (micro)albuminuria according to total protein intake adjusted for age, sex, impaired glucose tolerance/diabetes mellitus, hypertension and total homocysteine. The reference category was total protein intake 0.75 to 1.00 g/kg · day. Percentages of the population under study for each total protein intake range are presented. Error bars represent the lower or upper half of the 95% confidence intervals. *$P <$ 0.05, significantly different from the reference category. P for trend = 0.001.

respectively ($P = 0.2$). Additional adjustment for leukocyturia [that is, yes/no; present in 15.7% (93 of 594) of the subjects] also yielded similar results [OR (95% CI) 1.28 (1.01 to 1.61)], as did adjustment for the presence of cardiovascular disease (10.7%: 65 of 607), as defined elsewhere [8] OR 1.25 (1.05 to 1.50).

We evaluated possible modification by other risk factors of the effect of tHcy on risk of (micro)albuminuria and did not observe substantial differences among the following strata: sex, diabetes, hypertension, dyslipidemia, hypercholesterolemia and current smoking (data not shown). In addition, interaction terms between serum tHcy and these risk factors were not significant. Risk of (micro)albuminuria increased with increasing serum tHcy levels (Fig. 2). In none of the above analyses did log-transformation of tHcy result in a better fit (data not shown). Finally, although (micro)albuminuria is usually considered as a dichotomous variable, we also performed multiple linear regression analysis with log-transformed ACR as the (continuous) dependent variable. After adjustment for age, sex, diabetes, hypertension and renal function, ACR showed a highly significant association with tHcy ($P < 0.0001$).

The present study was too small to adequately investigate whether the relationship between microalbuminuria and cardiovascular disease can be explained to some extent by hyperhomocysteinemia (data not shown).

Protein intake correlated with creatinine clearance ($r = 0.2$; $P < 0.01$). An 0.1 g/kg · day increment of the daily animal or vegetable protein intake was associated, after adjustment for age, sex, classical risk factors and serum tHcy, with an increased risk of (micro)albuminuria [OR (95%CI); 1.22 (1.08 to 1.39) and 1.32 (1.07 to 1.62)]. Since these ORs did not differ substantially, we calculated the strength of the association between total protein intake and (micro)albuminuria to be OR 1.20 (1.08 to 1.32). After exclusion of known diabetic patients the OR was 1.18 (1.04 to 1.33). There was no evidence of interaction between hypertension or glycemic control and protein intake with regard to risk of (micro)albuminuria. Risk of (micro)albuminuria increased with increasing daily total protein intake (Fig. 3).

DISCUSSION

This is, to our knowledge, the first population-based study unequivocally showing that serum total homocysteine is positively associated with the presence of microalbuminuria independent of major determinants, that is, diabetes mellitus, hypertension, protein intake and renal function. For each 5 μmol/liter (about 1 SD) increase in serum tHcy level, the risk of microalbuminuria being present increased by about 30%. The result of the present study is in line with a few studies that reported a positive association between albuminuria and tHcy level [21, 22], but contradicts other studies [23, 24]. However, none of these studies adjusted for all major determinants of microalbuminuria and some [23, 24] used relatively small populations.

We chose two different approaches to investigate the nature of the association between tHcy and microalbuminuria. First, we analyzed the association with tHcy as a continuous variable, since there is evidence that the association of tHcy with risk of cardiovascular disease is graded [6, 25]. Second, we evaluated a possible dose-response relationship between microalbuminuria and several ranges of tHcy. Inspection of Figure 2 suggests that there might be a threshold (at 18 μmol/liter) above which an increased risk of microalbuminuria exists. Obviously the present study is not large enough to solve the question of whether the association between microalbuminuria and tHcy is graded or has a certain threshold.

Microalbuminuria is thought to be caused by increased glomerular albumin filtration as a result of decreased glomerular charge selectivity, size selectivity and/or increased intraglomerular pressure [26, 27], which regulation is affected by renal endothelial and mesangial cell function

[28, 29]. Mesangial cells have some properties in common with vascular smooth muscle cells [29]. Hyperhomocysteinemia may induce dysfunction of the vascular endothelium [30] and increase proliferation of vascular smooth muscle cells [31], possibly by increasing oxidative stress [32]. Therefore, it is conceivable that hyperhomocysteinemia is causally related to microalbuminuria through changes in renal endothelial and mesangial cell function and might thus be one of the factors that link the presence of microalbuminuria to an increased risk of atherothrombotic disease [1, 2].

We considered three sources of disease misclassification that may have resulted in bias of the association between hyperhomocysteinemia and persistent microalbuminuria. First, of the majority (72%) of subjects we collected only one urine sample to assess microalbuminuria. Repeated measurements would have improved the accuracy of classification of persistent microalbuminuria since there is a considerable day-to-day intra-individual variability of albumin excretion [33, 34]. Second, approximately 24% of all subjects classified as normoalbuminuric used antihypertensive medication. Antihypertensives, especially angiotensin-converting enzyme inhibitors, are likely to decrease urinary albumin excretion [35]. Finally, the presence of leukocyturia, which, insofar as it reflects urinary tract infection, might increase urinary albumin excretion. In all three cases, the possible disease misclassification was non-differential with regard to serum tHcy level. In general, non-differential disease misclassification would likely bias the findings toward effect attenuation (that is, underestimation of the strength of the association between hyperhomocysteinemia and microalbuminuria) [36].

The present study confirms and extends previous observations that tHcy and creatinine clearance are strongly associated [22]. An impaired renal function causes a substantial increase in the half-life of tHcy explained by a reduction in total body clearance [37, 38]. In addition, an impaired renal function is a risk factor for microalbuminuria. As the association between tHcy and microalbuminuria did not materially change after adjustment for serum creatinine or creatinine clearance (Table 2), we consider it improbable that impaired renal function confounded the association between tHcy and the presence of microalbuminuria. Finally, we cannot fully exclude that proximal tubular dysfunction in the presence of a normal glomerular filtration rate (GFR) results in both decreased albumin reabsorption (and thus microalbuminuria) and impaired tHcy metabolism, but this appears unlikely.

A poor folate, vitamin B_{12} and/or vitamin B_6 status can increase serum tHcy level [39]. Since we did not assess B vitamins and the present study is cross-sectional, we cannot rule out the possibility that low vitamin B levels may increase urinary albumin excretion or that microalbuminuria *per se* can raise serum tHcy levels, although this appears biologically implausible. Even though in an elderly population hyperhomocysteinemia, microalbuminuria and vitamin B deficiency frequently coexist, this does not explain the positive association we found between serum tHcy and microalbuminuria. Only when vitamin B deficiency would be the cause of both microalbuminuria and hyperhomocysteinemia, which seems unlikely with respect to microalbuminuria, could vitamin B deficiency have been a confounder of the association between tHcy and microalbuminuria. Serum tHcy levels can be lowered with an increased intake of B vitamins, particularly folate. A randomized clinical trial could thus support the hypothesis that tHcy might be a causal factor of microalbuminuria. Although the dose-response relationship we found between hyperhomocysteinemia and microalbuminuria does not necessarily support a causal relationship between tHcy and microalbuminuria, departures from expected biologic gradients do provide evidence against causation.

We investigated whether dietary protein intake confounded the association between tHcy and microalbuminuria. We found that dietary protein intake did not explain the relationship between tHcy and microalbuminuria. However, we did observe an increased risk of microalbuminuria with increasing consumption of total protein independent of serum tHcy levels. This observation is consistent with some [9, 40] but not all studies [41]. A high protein intake may result in an increased GFR and renal workload and therefore aggravate proteinuria [42]. In addition, it has previously been demonstrated that an animal compared to a vegetable protein diet, independently of the daily amount of protein intake, results in a higher GFR and urinary albumin excretion [43].

In the present study additional adjustment for animal or total protein intake, if anything, strengthened the association between serum tHcy with regard to risk of microalbuminuria. Taken together, it appears unlikely that hyperhomocysteinemia and high animal protein intake share a common causal pathway with regard to risk of microalbuminuria. Therefore, both appear important determinants of microalbuminuria.

We conclude that both hyperhomocysteinemia and a high protein intake are related to microalbuminuria independent of non-insulin-dependent diabetes mellitus and hypertension. Hyperhomocysteinemia may partly explain the link between microalbuminuria and increased risk of cardiovascular disease.

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