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Low fetuin-A levels are associated with cardiovascular death: Impact of variations in the gene encoding fetuin

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Low fetuin-A levels are associated with cardiovascular death: Impact of variations in the gene encoding fetuin.

Background. Vascular calcification is common among end-stage renal disease (ESRD) patients and a central characteristic of the atherosclerotic cardiovascular disease observed in dialysis patients. Fetuin-A, a circulating calcium-regulatory glycoprotein that inhibits vascular calcification, is associated with inflammation and outcome in dialysis patients. In the present study, we evaluated the association between fetuin-A, clinical phenotype, and outcome, as well as the impact of fetuin gene (AHSG) polymorphisms on the protein product and outcome.

Methods. In a cohort of 258 (161 males) ESRD patients starting renal replacement therapy [glomerular filtration rate (GFR) 6.8 ± 0.2 mL/min] aged 52 ± 1 years the following parameters were studied: presence of malnutrition (subjective global assessment), comorbidity [diabetes mellitus and clinical manifest cardiovascular disease (CVD)], carotid plaques ($N = 101$), hs-CRP, fetuin-A, S-albumin, interleukin (IL)-6, and single nucleotide polymorphisms (SNPs) in the AHSG gene ($N = 215$) at amino acid positions Thr248Met (C→T), Thr256Ser (C→G), Asp276Asn (G→A), and Arg317Cys (C→T).

Results. Both all-cause ($P < 0.001$) and cardiovascular ($P < 0.001$) mortality were associated with low fetuin-A levels independently of age, smoking, diabetes, S-albumin, CVD, and inflammation (CRP ≥10 mg/L). Inflamed (0.199 vs. 0.247 g/L; $P < 0.01$) and malnourished (0.207 vs. 0.262 g/L; $P < 0.05$) patients had significantly lower median fetuin-A than noninflamed and well-nourished ESRD patients, respectively. In a logistic regression model ($N = 101$), fetuin-A was significantly ($P < 0.05$) associated with the presence of carotid plaques independently of age, CVD, diabetes, S-albumin, gender, and inflammation. Significant correlations were observed between fetuin-A and both S-albumin (Rho = 0.30; $P < 0.0001$) and IL-6 (Rho = −0.21; $P < 0.01$). Patients with the AHSG 256Ser allele had lower serum fetuin-A levels, and higher all-cause and cardiovascular mortality rate if they were inflamed.

Conclusion. The present study shows that a low fetuin-A level is associated with malnutrition, inflammation, and atherosclerosis (carotid plaques), as well as with increased cardiovascular and all-cause mortality. Because the present study demonstrates an effect of variations in the AHSG gene on both circulating fetuin-A levels and outcome, this indicates that ESRD patients with the AHSG 256Ser allele are at risk of accelerated vascular calcification.

Key words: fetuin-A, vascular calcification, inflammation, malnutrition, genetic polymorphism, S-albumin.

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Premature atherosclerotic cardiovascular disease (CVD) is a leading cause of morbidity and mortality in patients with end-stage renal disease (ESRD) [1]. Although traditional Framingham risk factors such as hypertension, dyslipidemia, and diabetes mellitus (DM) may account for a large proportion of the excessive burden of CVD in this patient population, recent studies suggest that non-traditional risk factors, such as inflammation, oxidative stress, and vascular calcification, may also contribute [2].

Whereas ectopic mineral calcification in the vessel wall is not commonly observed in younger age groups in the general population, it is extremely common among ESRD patients and a central characteristic of the progressive atherosclerosis observed in this patient group [3, 4]. The prevalence and extent of vascular and valvular calcification and arterial stiffness are strong predictors of CVD and all-cause mortality in both hemodialysis (HD) [5] and peritoneal dialysis (PD) [6] patients. It is notable that although enhanced extraosseus calcification seems to be related mainly to enhanced $Ca \times PO_4$ ion product in serum and a high daily calcium intake, recent evidence suggests that extraossoeus calcification in ESRD is not only a passive degenerative process but also involves active inflammation [4, 7, 8]. Notably, also other inflamed patient groups, such as systemic lupus erythematosus (SLE) patients [9], have an increased coronary

calcification burden. There may be several reasons why a state of chronic inflammation may promote vascular calcification and, in particular, mediators and inhibitors, such as leptin [10], matrix-gla protein [11], tumor necrosis factor [12], bone morphogenetic protein [13], and osteoprotegerin [14] may be related to a process of accelerated vascular calcification.

Fetuin-A [also called alpha2-Heremans Schmid glycoprotein (AHSG)] is a circulating negative acute-phase glycoprotein [15] synthesized in adult human liver tissue [16] that inhibits ectopic $Ca \times PO_4$ ion precipitation and vascular calcification [17]. Because Shafer et al [18] have demonstrated that AHSG-deficient mice develop severe calcification of various organs, it has been suggested that an important clinical role of fetuin-A may be as an inhibitor of ectopic calcification. Indeed, Ketteler et al [19] measured fetuin-A levels in 312 prevalent HD patients and found that patients with low fetuin-A serum levels showed a significantly poorer survival than those with normal or high-normal values. Thus, low fetuin-A levels may link inflammation, accelerated atherosclerosis, and CVD in uremic subjects and could, at least in part, explain the high prevalence of vascular calcification in this population.

Although inflammation may be one important cause of low circulating fetuin-A levels, other factors may also contribute. Because it has been demonstrated that at least four nonsynonymous polymorphisms exist in the AHSG gene [20], genetic alterations may have an effect on the circulating amounts of this protein. One main objective of the present study was, therefore, to study the impact of genetic AHSG polymorphisms on circulating fetuin-A levels and outcome. Furthermore, the associations between fetuin-A and the phenotype of ESRD patients, in particular, the presence of inflammation, malnutrition, and atherosclerosis (carotid plaques) were evaluated.

METHODS

Study population

Two hundred and fifty-eight ESRD patients (161 males) with a mean age of 52 ± 1 years were evaluated shortly [glomerular filtration rate (GFR) 6.8 ± 0.2 mL/min] before the beginning of renal replacement therapy (RRT). Whereas the majority ($N = 249$) of the patients were Caucasian, three were of African and six of East-Asian origin. The patients were enrolled in an ongoing prospective study on predialysis patients, the results of which have been published in part elsewhere [21]. The study exclusion criteria were age >70 years, overt infectious complications, and unwillingness to participate in the study. Seventy-eight (30%) of the patients had DM. Ninety (35%) of the patients had a clinical history of cerebrovascular, cardiovascular, and/or peripheral vascular disease (grouped as CVD). Of the 90 patients, 25 had suffered from cerebrovascular disease (stroke), 55 of cardiovascular disease (acute myocardial infarction, angina pectoris, or patients that had undergone coronary artery bypass surgery); 27 had a history of peripheral ischemic atherosclerotic vascular disease, and four patients had a history of an aortic aneurysm. Mean systolic and diastolic blood pressures at the time of investigation were 151 ± 2 and 89 ± 1 mm Hg, respectively. Evaluation of smoking habits revealed that 133 patients were former or current smokers, whereas 125 were nonsmokers.

During the observation period (3.5 ± 0.2 years; range 0.1–9.0 years), 88 patients died; 61 due to complications related to CVD (cardiovascular $N = 43$, cerebrovascular $N = 14$, peripheral vascular disease $N = 3$, and aortic aneurysm $N = 1$), whereas 27 patients died of noncardiovascular, or unknown, causes (cancer $N = 7$, unknown cause $N = 7$, infectious complications $N = 7$, wasting $N = 4$; fatal car accident $N = 1$, and refusal of treatment $N = 1$). One hundred thirty-six of the patients started PD, whereas 118 patients started HD. Four patients received a kidney transplant before starting dialysis treatment, and one patient had not yet started RRT at the time of evaluation. One hundred and two patients received a kidney transplant subsequent to entering the study and were followed in the same way as those that did not receive a transplant. No significant differences in age (52 ± 1 vs. 52 ± 1 years), median fetuin-A (0.237 vs. 0.222 g/L), S-albumin (32.4 ± 0.7 vs. 32.9 ± 0.5 g/L), prevalence of clinical CVD (35 vs. 35%), prevalence of malnutrition (SGA >1) (32 vs. 36%), mean arterial blood pressure (110 ± 2 vs. 108 ± 2 mm Hg), observation time (3.7 ± 0.2 vs. 3.2 ± 0.2 years), or proportion of patients undergoing renal transplantation during follow-up (41 vs. 36%) were noted between patients starting HD or PD. Many patients were on antihypertensive medications, such as angiotensin-converting enzyme inhibitors ($N = 148$), beta-blockers ($N = 137$), and calcium-blockers ($N = 94$), as well as other commonly used drugs in ESRD, such as phosphate and potassium-binders, diuretics, and vitamins B, C, and D. Thirty-six patients (14%) were on statin treatment at the time of inclusion in the study. The median dose of erythropoietin was 4000 IU/week (range 0–21,000 IU/week). Two different control groups were studied. The control subjects for AHSG genotyping consisted of 207 unrelated Caucasians living in the Stockholm area (62% males; mean age 40 ± 1 years), whereas the control group for serum fetuin-A consisted of 70 healthy subjects (staff of a hospital) from the San Diego area (17 males; mean age 49 ± 2 years) with normal renal function (S-creatinine ranging between 0.2–1.2 mg/dL). The racial distribution was different to the Swedish population (44 Caucasians, 21 blacks, four Asians, and one Mexican). All sera were aliquoted and stored at lower than −20°C. The Ethics Committee of Karolinska Institutet approved the

study protocol at Karolinska University Hospital at Huddinge, Stockholm, and informed consent was obtained from all patients.

Laboratory methods

After an overnight fast, venous blood samples were taken for analysis of serum albumin, CRP, high-sensitivity (hs) CRP ($N = 250$), IL-6 ($N = 255$), serum calcium, serum phosphate, parathyroid hormone (PTH), and serum fetuin-A. In 131 of the patients (mean age 53 ± 1 years; 81 males), repeated measurements of fasting serum fetuin-A, S-albumin, and hs-CRP ($N = 67$) were performed after about 12 months of RRT (PD = 79 and HD = 52). The samples were kept frozen in -70°C if not analyzed immediately. GFR (corrected for body surface area) was estimated as the mean of urea and creatinine clearance. Serum fetuin-A levels were measured by a sandwich immunoenzymometric assay using two polyclonal human fetuin-A specific antibodies (Epitope Diagnostics, Inc., San Diego, CA, USA). Briefly, an affinity purified human fetuin-A specific polyclonal antibody was coated onto the well surface of standard 96-well microplate as solid-phase, and another affinity purified human fetuin-A specific antibody from different animal species was directly labeled with horseradish peroxidase (HRP) as assay tracer. After incubation, a sandwich of "solid-phase anti-fetuin antibody –sample fetuin-A–anti-fetuin HRP antibody" was formed, and after a washing step, the binding tracer antibody was detected by adding TMB substrate for color reaction. The lowest level of human fetuin-A in prediluted serum samples detected by this assay is 0.025 g/L, and the assay linear measurement range of human fetuin-A in prediluted serum sample is up to 7 g/L. The intra-assay variation is less than 5.5% and interassay variation less than 6.8%, depending on the sample concentration. CRP levels were measured using an immunonephelometric method (Tina-quant®; Boehringer-Mannheim/Hitachi, Ltd., Tokyo, Japan), hs-CRP by nephelometry, and S-albumin by the bromcresol purple method. Plasma IL-6 levels were measured by a high-sensitivity photometric enzyme-linked immunosorbent assay (ELISA) (Boehringer Mannheim, Mannheim, Germany). The plates were read using ELISA VERSAmax reader™ (Molecular Devices Corp., Sunnyvale, CA, USA), and the data were analyzed with the SoftmaxPRO® software (Molecular Devices Corp.). S-calcium, S-phosphate, and PTH were analyzed using routine methods. Subjective global assessment (SGA) was used to obtain an overall clinical estimate of malnutrition as previously described [22].

In the first 101 patients participating in this study, the right and left carotid arteries were examined with a duplex scanner (Ultramark 9HDI; Advanced Technology Laboratory, Bothwell, WA, USA) using a 5 to 10 MHz linear array transducer. The subjects were investigated in the supine position with the head slightly turned from the investigator. The same trained sonographer performed all scans. Carotid plaque was defined as a localized intima-media thickness of more than 1 mm, and at least a 100% increase in thickness compared with adjacent wall segments. Plaques were screened for in the common, internal, and external carotid arteries. Plaque occurrence was scored as the absence of plaques or the presence of unilateral and bilateral plaques (grouped together). The intraobserver variability (coefficient of variation) for intima-media thickness was 9% [23].

Genotyping

From a 5 mL EDTA sample of peripheral blood DNA was extracted using QIAamp® DNA kits (Qiagen, Valencia, CA, USA). Samples were stored at -20°C. Sequence amplification was performed by the polymerase chain reaction (PCR) on a PTC-225 Thermocycler (MJ Research, Inc., Cambridge, MA, USA). The PCR reaction volume was 50 μL, containing 20 to 50 ng of DNA, 10 pmol of each forward and reverse primer, 0.2 mmol/L of each dNTP, 0.3 U of DyNAzyme™ II (DNA Polymerase, Finnzymes, Espoo, Finland), 10 mmol/L of Tris-HCl, 1.5 mmol/L of $MgCl_2$, 50 mmol/L of KCl, and 0.1% Triton X-100. PCR primers were designed using the software Primer Designer 4 for Windows, version 4.1 (Scientific and Educational Software, Cary, NC, USA), and one primer in each primer pair was biotinylated. The Thr248Met polymorphism was amplified using forward primer 5′ biotin - GGCTTTTGTAAGGCAACACT-3′ and reverse primer 5′- GCAAGAATATTCAC GGAGCT -3′. The Thr256Ser and Asp276Asn polymorphisms were amplified in a single fragment using forward primer 5′ biotin -TCCTTTTTCCAGCCCGTGA -3′ and reverse primer 5′- TGCACCACTGTGCGT-GTTTT -3′. For amplification of the Arg317Cys polymorphism the same PCR primers were used, but in this case the reverse primer was biotinylated (GenBank accession no: AB038689). Sequencing primers were placed adjacent to the single nucleotide polymorphisms and were 5′- CCTGTGTTTGGAACACC -3′; 5′-GGTTGGGGCTGTGAG-3′; 5′- GGACGGAGGT-GCAT -3′ and 5′-GGCGCACTACGACC -3′ for AHSG Thr248Met, Thr256Ser, Asp276Asn, and Arg317Cys, respectively. All oligonucleotides were synthesized by Thermohybaid® (Ulm, Germany). The pyrosequencing reaction was performed on a PSQ96™ Instrument from Pyrosequencing AB (Uppsala, Sweden), as described previously [24].

Statistical analysis

Normally distributed variables are expressed as mean ± SEM, and non-normally distributed variables as

median and range, with $P < 0.05$ indicating significance. The two-sided unpaired Student t test was used for comparisons between two groups. Comparisons between groups for nominal variables were made by the Fisher exact test. Correlations were performed by linear regression or Spearman rank analysis and stepwise multiple regression analysis. Survival analyses were made with the Kaplan-Meier survival curve or the Cox proportional hazards model. The Cox proportional hazards model (the PHREG procedure in the SAS System Release 8.2, Cary, NC, USA) 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 hazard ratio (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. As the hazard ratio associated with age was found to be time-dependent, this covariate was dichotomized (>65 years). After the dichotomization there was no evidence that age (as a dichotomous variable) violated the proportional hazards assumption. No adjustments to presented P values were made for multiple comparisons [25]. Survival was measured from the day of examination until death or censoring, which was made at the end of the follow-up (December 3, 2003). No patient was lost to follow-up. Chi-square analysis was used to test the genotype distributions for Hardy-Weinberg equilibrium, and linkage disequilibrium was calculated according to Sham[26]. The statistical analysis was performed using statistical software SAS version 8.2 (SAS Institute, Inc., Cary, NC, USA).

RESULTS

Median fetuin-A was significantly ($P < 0.0001$) lower in 258 ESRD patients (0.225 g/L; range 0.026–0.926 g/L) compared to 70 healthy controls (0.549 g/L; range 0.350–0.950 g/L). Demographic and clinical data of the ESRD population with regard to clinically manifest CVD are presented in Table 1. As expected, 90 patients with clinical manifest CVD were older (60 ± 1 vs. 48 ± 1 years; $P < 0.0001$), and more often had DM (48 vs. 21%; $P < 0.0001$), malnutrition (53 vs. 24%; $P < 0.0001$), and evidence of inflammation (54 vs. 28%; $P < 0.0001$) than 168 patients with no clinical signs of CVD. As expected, the prevalence of former/current smokers versus nonsmokers (68 vs. 43%) was higher in the CVD patient group. Moreover, ESRD patients with CVD also had carotid plaques more often (92 vs. 59%; $P < 0.001$) than patients without clinically manifest CVD. Whereas median IL-6 (9.5 vs. 5.0 pg/mL; $P < 0.0001$) and hs-CRP (13.0 vs. 4.2 mg/L; $P < 0.0001$) were significantly elevated, S-albumin levels (30.7 ± 0.7 vs. 33.8 ± 0.5 g/L; $P < 0.001$) were lower in the CVD patient group. The difference in median fetuin-A level (0.204 vs. 0.237 g/L) between CVD and non-CVD patients did not attain full statistical significance. Seventy-eight diabetic patients had a significantly lower median fetuin-A (0.191 vs. 0.240 g/L; $P < 0.01$) than 180 non-diabetic ESRD patients. No differences in the median fetuin-A level were noted between nonsmokers and former/current smokers (0.247 vs. 0.217 g/L), or between males and females (0.225 vs. 0.223 g/L).

Eighty-eight malnourished (SGA ≥2) patients had significantly lower (0.207 vs. 0.262; $P < 0.05$) fetuin-A levels than 160 ESRD patients with no signs of malnutrition (SGA 1). Seventy-two patients with carotid plaques had significantly lower median fetuin-A (0.219 vs. 0.321 g/L; $P < 0.001$) and S-albumin (32.4 ± 0.8 vs. 35.9 ± 1.0 g/L; $P < 0.05$) levels than 29 patients with no carotid plaques (Fig. 1). In a logistic multiple regression model (total R^2 0.47) including age (≥median 54 years), gender, DM, CVD, evidence of inflammation (CRP ≥10 mg/L), low albumin levels (≤33 g/L), and fetuin-A (quartiles) only age (OR 40.7; 95% CI 4.4–378.2; $P < 0.001$) and fetuin-A (OR 2.2; 95% CI 1.1–4.0; $P < 0.05$) were significantly associated with the presence of carotid plaques.

Ninety-one patients with signs of inflammation (CRP ≥10 mg/L) had significantly ($P < 0.01$) lower median (0.199 vs. 0.247 g/L) fetuin-A than 167 patients with CRP <10 mg/L. Thirty-five patients with CRP ≥10 mg/L with no signs of malnutrition (SGA 1) had a median fetuin-A (0.215 vs. 0.197 g/L) not significantly different from 51 patients with CRP ≥10 mg/L and malnutrition (SGA >1). Significant correlations were observed between fetuin-A and both age (Rho = −0.22; $P < 0.001$) and S-albumin (Rho = 0.30; $P < 0.0001$) (Fig. 2). Significant negative correlations were observed between fetuin-A and both IL-6 (Rho = −0.21; $P < 0.01$) and hs-CRP (Rho = −0.14; $P < 0.05$). No significant correlation (Rho = 0.10) was observed between GFR and fetuin-A in 225 of the patients. A stepwise (forward) multiple regression model analysis, correcting for the impact of age, gender, DM, and S-albumin revealed independent (point estimates ± SE) relationships (total R^2 0.11) only between age (0.08 ± 0.03; $P < 0.05$) and S-albumin (-0.14 ± 0.03; $P < 0.001$) and fetuin-A levels (log-transformed).

Median fetuin-A level decreased significantly ($P < 0.001$) from 0.222 g/L (range 0.026–0.876 g/L) to 0.198 g/L (range 0.065–0.458 g/L) during 12 months of RRT. No significant difference in change (Δ) of fetuin-A levels was observed between 79 patients treated by PD (-0.069 ± 0.019 g/L) and 52 patients treated by HD

Table 1. Clinical characteristics of 258 incident end-stage renal disease patients with or without clinical evidence of cardiovascular disease

	No CVD	CVD	Significance
Number	168	90	
Age *years*	48 ± 1	60 ± 1	<0.0001
Males	59%	69%	NS
Smokers (former and current)	43%	68%	<0.0001
Diabetes mellitus	21%	48%	<0.0001
Malnutrition (SGA >1)[a]	24%	53%	<0.0001
Inflammation (CRP ≥10 mg/L)	28%	54%	<0.0001
Carotid plaques[b]	59%	92%	<0.001
Body mass index kg/m^2	24.3 ± 0.4	25.1 ± 0.5	NS
S-calcium *mmol/L*	2.53 ± 0.02	2.56 ± 0.03	NS
S-phosphate *mmol/L*	1.96 ± 0.05	1.79 ± 0.07	<0.05
Parathyroid hormone *ng/L*	286 ± 23	281 ± 41	NS
hs-CRP *mg/L*[c,e]	4.2 (0.2–105.0)	13.0 (0.3–218.0)	<0.0001
Interleukin-6 *pg/mL*[d,e]	5.0 (0.8–46.5)	9.5 (1.4–112.0)	<0.0001
S-albumin *g/L*	33.8 ± 0.5	30.7 ± 0.7	<0.001
Fetuin-A *g/L*[e]	0.237 (0.026–0.926)	0.204 (0.046–0.758)	NS

SGA, subjective global assessment; CRP, C-reactive protein.
[a]$N = 248$; [b]$N = 101$; [c]$N = 250$; [d]$N = 255$; [e]median and range.

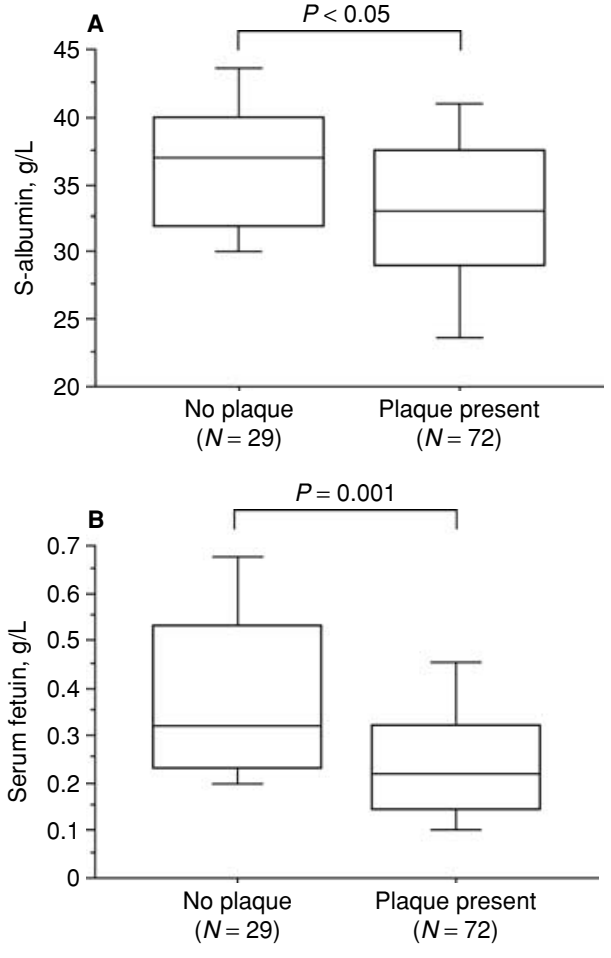

Fig. 1. Box plots showing serum albumin (*A*) and fetuin-A (*B*) levels in 101 ESRD patients with and without carotid plaques.

(−0.071 ± 0.024 g/L). No significant correlations were observed between Δ fetuin-A and either ΔS-albumin (Rho = 0.12) or Δhs-CRP (Rho = −0.19) during 12 months of RRT.

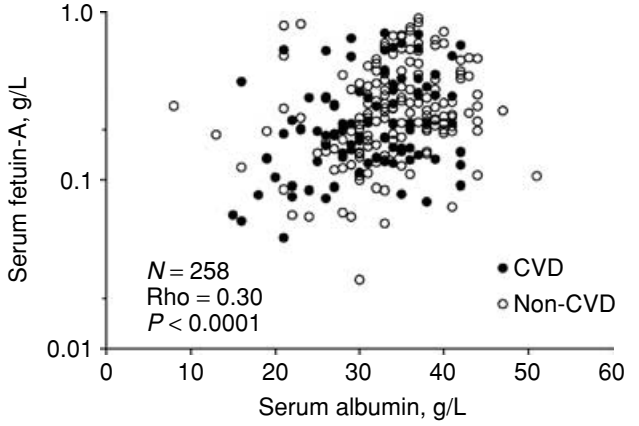

Fig. 2. The correlation (Rho = 0.30; $P < 0.0001$) between fetuin-A and serum albumin in 258 ESRD patients.

By using Kaplan-Meier survival curves we assessed the association between fetuin-A concentrations, divided into quartiles with respect to low (0.026–0.153 g/L; $N = 65$), medium low (0.154–0.224 g/L; $N = 64$), medium high (0.225–0.362 g/L; $N = 64$), and high (0.363–0.928 g/L; $N = 65$) serum concentrations, and noted significantly increased all-cause (log-rank 34.2; $P < 0.0001$) and CVD (log-rank 23.4; $P < 0.0001$) mortality in patients with low amounts of fetuin-A (Fig. 3). When patients were divided according to median fetuin-A (≥0.225 g/L), the adjustment for potential confounding factors (age, gender, DM, S-albumin, inflammation, CVD, and smoking) using Cox proportional hazards model revealed a significant (likelihood ratio 118.1; $P < 0.0001$) difference in survival between the two groups (Fig. 4). The Cox proportional hazard model was used to adjust both all-cause and cardiovascular mortality for age (≥65 years), gender, DM, CVD, smoking habits (former/current smokers vs. nonsmokers), mode of RRT (PD vs. HD), $Ca \times PO_4$

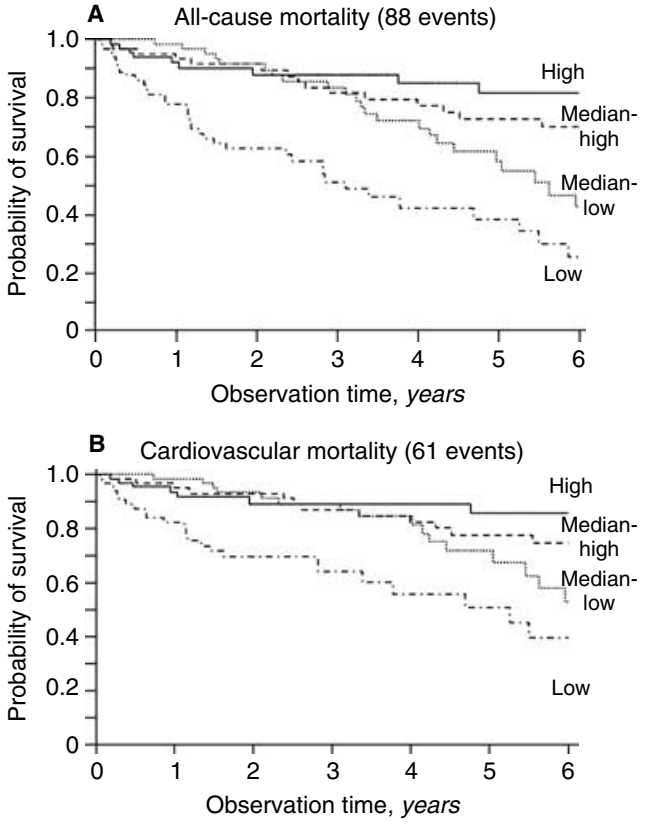

Fig. 3. Kaplan-Meier curves showing differences in all-cause (log-rank 34.2; $P < 0.0001$) (*A*) and cardiovascular (log-rank 23.4; $P < 0.0001$) (*B*) mortality in 258 ESRD patients starting renal replacement therapy according to basal serum fetuin-A levels (quartiles).

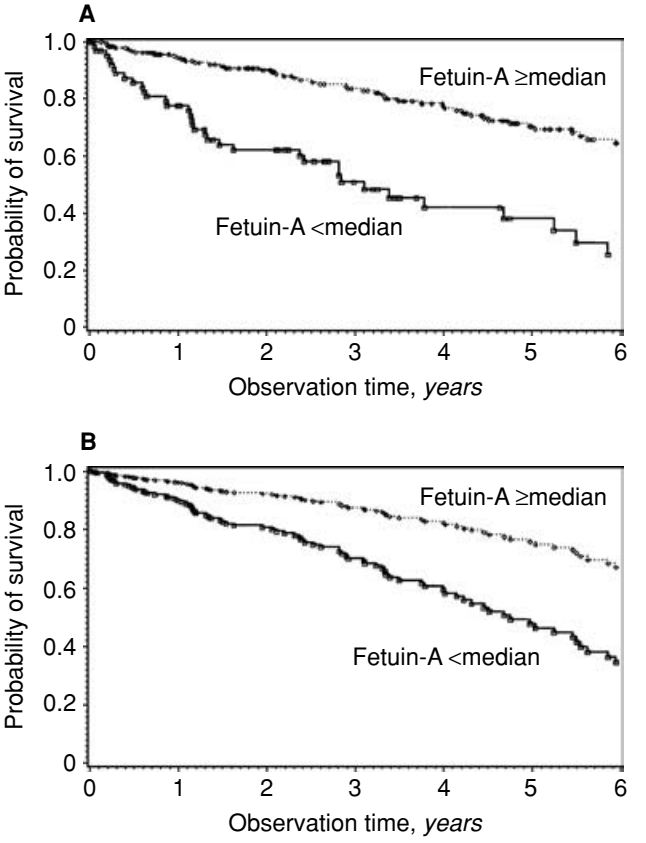

Fig. 4. Kaplan-Meier curves showing unadjusted (log-rank 19.6; $P < 0.0001$) (*A*) and adjusted (likelihood ratio 118.1 $P < 0.0001$) all-cause mortality (*B*) in 258 ESRD patients according to median fetuin-A levels.

ion product (≥median), hypoalbuminemia (≤33 g/L), inflammation (CRP ≥10 mg/L), and fetuin-A (quartiles). By univariate analysis all variables, except gender, mode of RRT, and $Ca \times PO_4$ ion product, were significantly associated with both all-cause and CVD mortality as shown in Table 2. When we did a multivariate Cox regression analysis including variables that were significant in the univariate analysis, fetuin-A quartiles remained significantly associated to both all-cause ($P < 0.001$) and CVD mortality ($P < 0.001$), whereas both CRP and S-albumin lost their association to outcome (Table 2). If fetuin-A was not included in the model, the association between hypoalbuminemia (≤33 g/L) and all-cause mortality was significant (RR 1.67; CI 1.03–2.71; $P < 0.05$) even after correction for CVD, DM, inflammation, and age. We also tested the impact of transplantation as a time-dependent variable, but it did not influence either all-cause or CVD mortality in the Cox regression model.

The distribution of the AHSG Thr248Met (C→T), Thr256Ser (C→G), Asp276Asn (G→A), and Arg317Cys (C→T) genotypes was investigated in 215 of the patients. No differences in the genotype distribution of the four AHSG SNPs were noted between the ESRD patients and the 209 controls (data not shown). The genotype distribution for the Thr248Met and the Thr256Ser genotypes were in total linkage disequilibrium (LD) ($D' \geq 0.999$) in both patients and controls. Thus, in the following, only data for the Thr256Ser polymorphism are given. Since all patients tested for the Asp276Asn polymorphism were Asp/Asp homozygotes, no further analyses were done for this variation. No significant differences in the distribution between males versus females, patients with and without signs of inflammation, CVD versus non-CVD, and DM versus non-DM were noted for the Thr256Ser and Arg317Cys variations. However, the AHSG Thr256Ser polymorphism was associated with marked differences (Thr/Thr 0.276 g/L, Thr/Ser 0.217 g/L; Ser/Ser 0.151 g/L; $P < 0.0001$) in median fetuin-A levels. A stepwise (forward followed by backward) multiple regression model analysis ($N = 215$), correcting for the impact of age, gender, DM, and S-albumin, revealed independent (point estimate ± SE) relationships (total R^2 0.17) between the Thr256Ser polymorphism (0.09 ± 0.03; $P < 0.01$), S-albumin (-0.15 ± 0.04; $P < 0.001$), DM (0.09 ± 0.04; $P < 0.05$), age (0.07 ± 0.03; $P = 0.05$), and fetuin-A levels (log-transformed). When patients were divided according to the presence of inflammation or not, both uninflamed ($P < 0.001$) and inflamed ($P < 0.05$) patients with

Table 2. Unadjusted and adjusted hazard ratios for all-cause and cardiovascular mortality in patients starting renal replacement therapy

All-cause mortality Variable	Unadjusted relative risk (95% CI)	*P* value	Adjusted relative risk (95% CI)	*P* value
Cardiovascular disease	5.49 (3.53–8.55)	<0.001	3.36 (2.09–5.41)	<0.001
Fetuin-A (low vs. high)	3.23 (2.10–4.95)	<0.001	2.58 (1.64–4.07)	<0.001
Age (≥65 years)	4.54 (2.94–7.00)	<0.001	2.54 (1.56–4.14)	<0.001
Diabetes mellitus	2.66 (1.75–4.05)	<0.001	1.75 (1.13–2.72)	<0.05
Inflammation (CRP ≥10 mg/L)	3.05 (2.00–4.67)	<0.001	1.52 (0.96–2.43)	NS
S-albumin (≤33 g/L)	2.61 (1.65–4.13)	<0.001	1.32 (0.80–2.20)	NS
Smoking (former/current vs. no smoking)	1.78 (1.16–2.73)	<0.01	1.45 (0.92–2.30)	NS
High Ca × PO_4 product (>median)	1.38 (0.82–2.31)	NS	–	–
Mode of RRT (PD vs. HD)[a]	1.09 (0.71–1.67)	NS	–	–
Male gender	1.30 (0.83–2.02)	NS	–	–
Cardiovascular mortality Variable	Unadjusted relative risk (95% CI)	*P* value	Adjusted relative risk (95% CI)	*P* value
Cardiovascular disease	7.87 (4.46–13.89)	<0.001	4.87 (2.66–8.92)	<0.001
Fetuin-A (low vs. high)	3.18 (1.89–5.35)	<0.001	2.63 (1.51–4.59)	<0.001
Diabetes mellitus	3.75 (2.26–6.21)	<0.001	2.47 (1.45–4.20)	<0.001
Age (≥65 years)	4.11 (2.46–6.86)	<0.001	2.23 (1.26–3.95)	<0.01
Inflammation (CRP ≥10 mg/L)	3.13 (1.88–5.21)	<0.001	1.57 (0.90–2.73)	NS
S-albumin (≤33 g/L)	2.21 (1.29–3.77)	<0.01	1.11 (0.61–2.01)	NS
Smoking (former/current vs. no smoking)	1.77 (1.06–2.96)	<0.05	1.26 (0.73–2.19)	NS
High Ca × PO_4 product (>median)	1.31 (0.71–2.40)	NS	–	–
Mode of RRT (PD vs. HD)[a]	1.19 (0.71–1.99)	NS	–	–
Male gender	1.32 (0.77–2.25)	NS	–	–

Abbreviations: RRT, renal replacement therapy; PD, peritoneal dialysis; HD, hemodialysis.
[a] $N = 254$.

the 256Ser/Ser genotype had significantly lower fetuin-A levels than 100 patients with the Thr/Thr genotype (Fig. 5). Moreover, patients with CRP ≥10 mg/L and the 256Thr/Ser genotype had significantly ($P < 0.01$) lower fetuin-A levels than patients with no signs of inflammation with the same alleles. Near-significant differences in all-cause (log rank 2.8; $P = 0.09$) and CVD (log rank 3.2; $P = 0.07$) mortality were observed between 116 ESRD patients carrying the 256Ser allele (Thr/Ser and Ser/Ser) and 99 non-Ser allele-carrying patients. If patients were further divided according to the presence of inflammation, inflamed (median hs-CRP 20 mg/L) patients carrying the 256Ser allele (Thr/Ser and Ser/Ser) had a significantly higher mortality rate ($P < 0.05$) than inflamed (median hs-CRP 25 mg/L) non-Ser allele (Thr/Thr) patients (Fig. 6). The difference between the two groups of patients was even more pronounced ($P < 0.01$) after two years of RRT (Fig. 6).

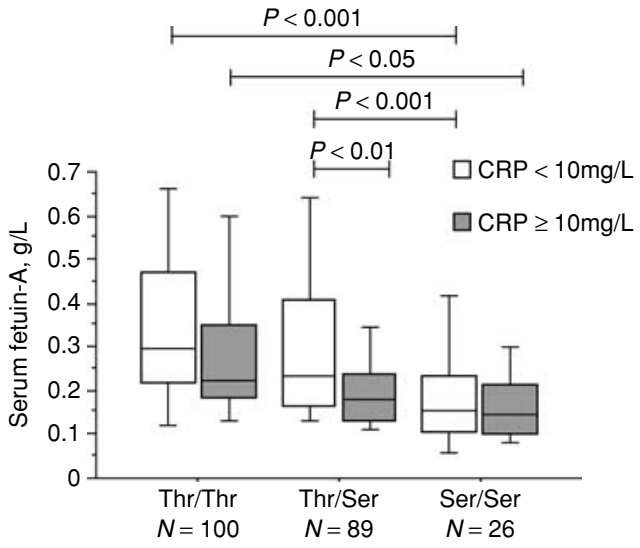

Fig. 5. Box plot showing serum fetuin-A levels in 215 ESRD patients according to the AHSG Thr256Ser polymorphism and evidence of inflammation (CRP ≥10 mg/L).

DISCUSSION

The present study confirms that low serum fetuin-A levels are found in ESRD patients, and that a reduction of this liver-derived glycoprotein is associated with inflammation and predicts both all-cause and cardiovascular mortality in ESRD patients [19]. Our data extend the previous findings by Ketteler et al [19] and show that even after adjustments for traditional and nontraditional risk factors, low s-fetuin-A is associated with poor outcome (Fig. 4). Thus, measurement of circulating fetuin-A may, in ESRD patients, add valuable prognostic information. Although it has repeatedly been shown that low albumin levels are associated with both mortality [27] and cardiac disease [28, 29] in ESRD patients, the reasons for this association has not been established. In the present study we found that the significant association between S-albumin and mortality was lost when fetuin-A was

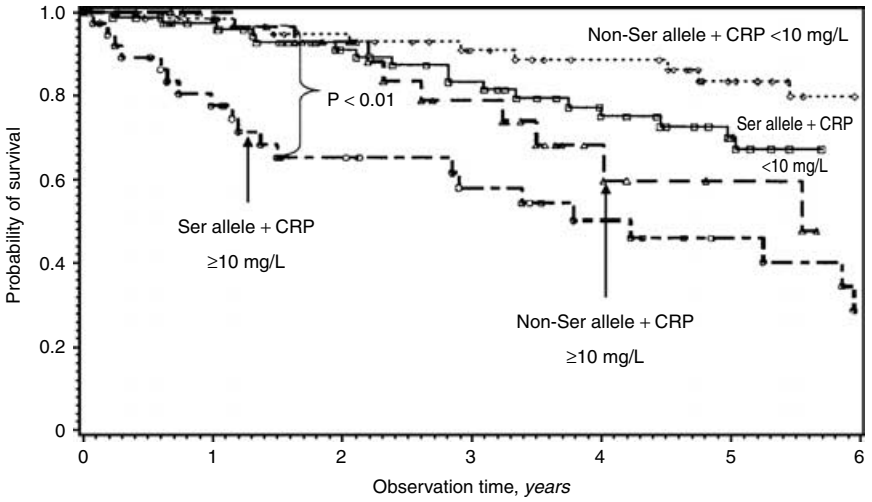

Fig. 6. Kaplan-Meier curve showing differences (log-rank 27.6, $P < 0.0001$) in all-cause mortality in ESRD patients divided into four groups according to the presence (CRP ≥10 mg/L) or absence of inflammation and the presence or absence of the amino acid serine at position 256.

introduced into the Cox model. Thus, our results suggest that the well-documented association between S-albumin and vascular disease may be explained, at least in part, by fetuin-A deficiency promoting vascular calcification (Fig. 2).

One aim of the present study was to evaluate the relationships between circulating fetuin-A levels and the phenotype of ESRD patients. Our results show a significantly lower median fetuin-A level in ESRD patients with evidence of inflammation, which confirms previous findings [19]. In addition, we found a significant inverse association between fetuin-A and both IL-6 and hs-CRP, showing that fetuin-A acts as a negative acute-phase reactant [15]. It has been reported that interleukin-1β down-regulates the fetuin-A hepatic mRNA level [30]. Moreover, in LPS-challenged mice hepatic fetuin-A mRNA is markedly down-regulated [31]. As fetuin-A may have important functions in inflammation, such as limitation of cytokine production by macrophages [32] and protection against TNF [33], further studies are needed to investigate the role of fetuin-A in the inflamed ESRD patient. The present data also demonstrate a lower median fetuin-A level in malnourished ESRD patients—a finding not surprising in view of the strong associations between inflammation and malnutrition (i.e., wasting) in ESRD [21]. Indeed, a study by Wang et al [7] demonstrated an association of inflammation and malnutrition with cardiac valve calcification in PD patients. Whether poor nutritional intake per se may affect fetuin production in the liver remains to be determined. However, as we found no significant difference in median fetuin-A levels comparing ESRD patients with CRP ≥10 mg/L with and without signs of malnutrition, respectively, we find it likely that an inflammatory reaction is the major cause of low fetuin-A levels also in malnourished patients. The present study also showed an association between low fetuin-A levels and the presence of carotid plaques, independent of established risk factors, such as age, CVD, DM, S-albumin, and inflammation. This finding supports preliminary data by Moe et al [abstract; Moe et al, *J Am Soc Nephrol* 14:692A, 2003], who found a correlation between electron beam computer tomography (EBCT) coronary calcifications and low fetuin-A levels in 77 ESRD patients. Although it is well established that medial vascular calcification is a common cell-mediated process that predicts cardiovascular mortality in DM, the pathogenesis of this phenomenon is not well understood [34]. Clearly, as we found significantly lower fetuin-A levels in diabetic ESRD patients compared to nondiabetic ESRD patients, further studies should be done to clarify the role of this circulating calcification inhibitor in the accelerated calcification process observed in this patient group.

In the prospective part of the study we found that 12 months of RRT was associated with a small, but significant, reduction of serum fetuin-A levels. This finding may indirectly support the suggestion that the process of vascular calcification is accelerated by dialysis treatment [35]. Indeed, whereas Spiegel et al [36] have reported that coronary and aortic calcification was not a very common phenomenon in patients new to HD, Ketteler et al [19] reported higher coronary calcification scores in patients on long-term dialysis compared to short-term dialysis. Moreover, Moe et al [37] demonstrated that duration of dialysis (vintage) and age were the only factors that correlated with coronary calcification. Thus, it is tempting to speculate that decreasing fetuin-A levels during RRT may be one reason for accelerated vascular calcification. As neither mode of dialysis therapy nor changes in S-albumin or CRP levels were associated with the decline in fetuin-A levels, further studies are needed to investigate if other factors, such as changes in residual renal function, acid-base balance, or nutritional changes may cause a decline of fetuin-A levels during RRT.

A novel finding of the present study was the impact of genetic variations of the AHSG gene on circulating fetuin-A levels in ESRD patients. The patients carrying the 256Ser allele had lower fetuin-A levels than 256Thr allele carriers (Fig. 5). It is notable that the presence of inflammation had an inhibitory effect on fetuin-A levels in patients carrying the 256Thr allele. Considering the putative proatherogenic effects of low circulating fetuin-A levels in ESRD patients, it could be hypothesized that a patient with a genetic propensity for low fetuin-A levels (i.e., patients carrying the AHSG 256Ser allele) would have a higher risk for cardiovascular premature death if they are subjected to a chronic inflammatory process. Indeed, as 256Ser allele carriers with signs of inflammation had a higher mortality (especially at 2 years of follow-up) than non-256Ser carriers with signs of inflammation, this polymorphism seems to enhance the mortality risk associated with inflammation (Fig. 6). Our finding suggests a significant gene-environment interaction, where the involvement of inflammatory processes may further enhance the negative effects of low circulating fetuin-A levels. A number of recent studies have highlighted the importance of exposure to environmental influences for the pathogenesis of complex disorders, such as insulin resistance, DM, and CVD [38, 39], where the expression of the final phenotype(s) may depend on complex gene-environment interactions [40]. Clearly, additional studies are needed to evaluate if AHSG 256Ser allele carriers are a group of ESRD patients particularly prone to develop vascular calcification during dialysis treatment, and would benefit from different therapeutic strategies and targets

Several limitations of the present study should be considered. First, no evaluation of coronary calcification score (using EBCT or spiral CT) was performed. Instead, we used carotid plaques as a surrogate marker of coronary atherosclerosis and calcification. However, carotid ultrasonography has been shown to be a useful diagnostic method of coronary heart disease in a high prevalence population [23]. Moreover, a recent study has demonstrated that carotid plaque scores were strongly correlated with coronary artery calcification scores calculated by EBCT in 79 HD patients [41]. As carotid duplex sonography was available only in a subset ($N = 101$) of the patients, a larger patient group is needed to confirm the relationship between fetuin-A and this surrogate marker of coronary heart disease. However, in the present study no differences in either age, fetuin-A, S-albumin, prevalence of inflammation, or $Ca \times PO_4$ were noted between the patients in whom the presence of carotid plaques was evaluated and the 157 patients in whom it was not (data not shown). Second, as the association between clinically manifest CVD and median fetuin-A level was of only borderline significance, this may support the notion that the use of a clinical definition of CVD, as used in many previous clinical studies, may be inappropriate in defining the true prevalence of vascular disease in ESRD patients [2]. Third, only one basal determination of hs-CRP, fetuin-A, and IL-6 was used in the present study, which may be problematic since inflammatory biomarkers vary with time [42]. Fourth, the racial and gender distribution differed between ESRD patients and controls; two putative factors that could have influenced the observed difference in fetuin-A levels. Finally, the circulating serum levels of fetuin-A are lower in this study compared to the serum levels previously reported [19]. Although differences in the study population may partly contribute to the observed difference, it should be noted that whereas Ketteler et al [19] used a radioimmunoassay for measuring human fetuin-A (which has only one raw antiserum and thus may have some cross reaction to other serum proteins, such as alpha-1 antichymotrypsin), we used a new fetuin assay using two specific antibodies for fetuin and a sandwich assay method, which is more sensitive and specific.

CONCLUSION

The present study shows that a low level of fetuin-A is a robust inflammation and malnutrition-related mortality risk factor in ESRD patients receiving RRT. As patients with carotid plaques had a markedly lower fetuin-A level, this supports the hypothesis that low fetuin-A may promote a process of accelerated atherosclerosis and vascular calcification. The present study also demonstrates that the genetic AHSG Thr256Ser variation has a major impact on circulating serum fetuin-A levels and outcome, especially in patients with evidence of inflammation. Clearly, if this finding can be confirmed in another cohort, prospective studies are needed to elucidate if chronically inflamed ESRD patients carrying the AHSG 256Ser allele are particularly prone to develop accelerated vascular calcification during RRT.

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