Original Article

Fetuin-A and kidney function in persons with coronary artery disease—data from the heart and soul study

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Abstract

Background. Fetuin-A is a serum protein that inhibits ectopic vascular calcification and is present in lower concentrations in end-stage renal disease than in healthy controls. Whether fetuin-A concentrations are also lower in the setting of mild-to-moderate chronic kidney disease (CKD) is unknown.

Methods. We evaluated the associations of several parameters of kidney function including measured 24 h urinary creatinine clearance (CrCl), estimated glomerular filtration rate (GFR) by the Mayo Clinic quadratic GFR equation (qGFR), serum cystatin-C concentrations, and urinary albumin-to-creatinine ratio with serum fetuin-A concentrations in 970 outpatients with coronary artery disease. We used general linear models to determine the adjusted mean fetuin-A concentrations within each kidney function category.

Results. The mean age of the study sample was 67 years, 82% were male, 71% had hypertension and 26% had diabetes mellitus. In adjusted analysis, we observed no significant differences in mean fetuin-A concentrations across groups defined by CrCl, qGFR, or albumin-to-creatinine ratio groups. For example, adjusted mean fetuin-A concentrations were 0.66 g/l in participants with CrCl > 90, 60–90 and 45–60 ml/min/1.73 m2, and 0.65 g/l in participants with CrCl < 45 ml/min/1.73 m2. Higher serum cystatin-C (indicating worse kidney function) was associated with higher adjusted mean serum fetuin-A concentrations (lowest quartile 0.62 g/l, highest quartile 0.68 g/l; P for trend < 0.001).

Conclusions. Among ambulatory patients with coronary artery disease, there is no evidence that mild-to-moderate CKD is associated with lower concentrations of serum fetuin-A compared with persons with normal renal function. The mechanisms explaining the association between CKD and vascular calcification remain elusive.

Keywords: calcium; chronic kidney disease; fetuin-A; alpha-2-Heremans-Schmid-glycoprotein; vascular calcification; phosphorus

Introduction

Patients with end-stage renal disease (ESRD) and chronic kidney disease (CKD) experience cardiovascular disease risk that is much higher than that in age- and sex-matched populations with normal kidney function [1–3]. Several epidemiological studies have demonstrated accelerated vascular calcification in CKD and ESRD [4–6]. Vascular calcification was previously thought to be a passive process aggravated by hyperphosphataemia and hypercalcaemia and controlled adequately with careful attention to mineral balance [7,8]. However, accumulating evidence suggests that vascular calcification is a regulated process affected by intra- and extra-cellular mechanisms as well as serum-based proteins [9–11].

Because human serum is supersaturated with respect to calcium and phosphorus, the existence of serum-based precipitation inhibitors has long been postulated. Human fetuin-A (alpha-2-Heremans Schmid glycoprotein), a protein produced by the liver and secreted into serum in high concentrations (~0.5–1.0 g/l), is a major serum-based inhibitor of vascular calcification and accounts for roughly 50% of the inhibition of calcium and phosphorous precipitation [12,13]. Serum concentrations of fetuin-A are depressed in patients with ESRD, and lower serum concentrations were independently associated with risk of cardiovascular and all-cause mortality in this population [14].
Whether serum fetuin-A concentrations are associated with kidney function, and whether the protein acts as a key calcification inhibitor in mild-to-moderate CKD is unknown. In this cross-sectional study, we evaluated the association of four parameters of kidney function [the Mayo Clinic quadratic glomerular filtration rate (qGFR), 24 h urinary creatinine clearance (CrCl), serum cystatin-C and urinary albumin-to-creatinine ratio] with serum fetuin-A concentrations in a well-characterized cohort of 970 persons with stable coronary artery disease. We hypothesized that serum fetuin-A concentrations would be directly related to the kidney function (and inversely related to proteinuria), with concentrations intermediate to those previously described in normal and uraemic populations.

Methods

Study participants

The Heart and Soul Study is a prospective cohort study designed to investigate the influence of psychosocial factors on progression of coronary artery disease. Methods have been described previously [15–17]. Briefly, participants were recruited from outpatient clinics in the San Francisco Bay Area if they met one of the following inclusion criteria: (i) history of myocardial infarction; (ii) angiographic evidence of > 50% stenosis in one or more coronary vessels; (iii) evidence of exercise-induced ischaemia by treadmill or nuclear testing; (iv) history of coronary revascularization or (v) documented diagnosis of coronary artery disease by an internist or cardiologist. Participants were excluded if they were not able to walk one block, had a myocardial infarction within the past 6 months or were likely to move out of the area within 3 years.

The study protocol was approved by the following Institutional Review Boards: the University of California San Francisco Committee on Human Research; the Research and Development Committee at the San Francisco VA Medical Center; the Medical Human Subjects Committee at Stanford University; the Human Subjects Committee at the VA Palo Alto Health Care System and the Data Governance Board of the Community Health Network of San Francisco. All participants provided written informed consent.

Between September 2000 and December 2002, a total of 1024 participants enrolled and underwent a daylong baseline study appointment that included a medical history interview, a physical examination, an exercise treadmill test with stress echocardiogram and a comprehensive health status questionnaire. Fasting (12 h) serum samples were obtained and frozen at −70°C. Subjects for whom frozen serum was not available were excluded, resulting in a final sample size of 970 subjects for the present analysis.

Parameters of kidney function

Each participant provided a 24 h urine collection and CrCl was determined as previously described [16]. CrCl was calculated using the following formula: Urine Cr (mg/dl) × 24 h urine volume (dl)/Serum Cr (mg/dl) × 1440 (min/day). GFR was estimated by means of the Mayo Clinic qGFR equation proposed by Rule and colleagues [18] qGFR = exp [1.911 + 5.249/SCr − 2.114/SCr² − 0.00686 × Age − 0.205 (if female)], by replacing SCr = 0.8 if SCr < 0.8 mg/dl. This measure of the kidney function was used in lieu of the Modification of Diet in Renal Disease (MDRD) Study formula because a substantial fraction of our study population had normal or near normal kidney function, and the MDRD equation has been shown to consistently underestimate kidney function in this setting [18]. Age and sex were obtained from the questionnaire. Serum creatinine was determined by the Jaffe reaction from venous samples obtained during the study visit.

Urine albumin and creatinine were measured by nephelometry and the Jaffe method, respectively. Spot urine albumin-to-creatinine ratios (mg albumin/g creatinine) were calculated for all participants.

Serum cystatin-C concentrations were measured from venous samples collected during the study visit using a BNII nephelometer (Dade Behring, Inc., Deerfield, IL) with a particle-enhanced immunonephelometric assay (N Latex Cystatin-C, Dade Behring, Inc.) [19]. Monoclonal antibodies to cystatin-C were coated on polystyrene particles which agglutinate to increase the intensity of scattered light in proportion to the concentration of cystatin-C. The assay range is 0.195 to 7.330 mg/l; the reference range for young healthy persons ranges from 0.53 to 0.95 mg/l. The intra-assay coefficient of variation ranges from 2.0 to 2.8%, and the inter-assay coefficient of variation ranges from 2.3 to 3.1%.

Serum fetuin-A

Serum fetuin-A was measured with a BNII nephelometric assay. Serum samples were centrifuged (60 min at 15000g) and diluted 4-fold with phosphate buffered saline (400 µl)(N Diluent, Dade Behring Holdings, Liederbach, Germany). Serum was exposed to a polyclonal rabbit anti-human fetuin-A antibody identical to that used in the ELISA method previously described [14]. Particles agglutinated to increase the intensity of scattered light proportionally to the amount of fetuin-A in the sample. A control solution of purified serum fetuin-A powder (Boehringer Mannheim GmbH, Mannheim; Dade Behring, Marburg, Germany) was used to prepare a serial dilution curve, and serum concentrations of fetuin-A were calculated by regression analysis of standard curves. The assay was evaluated in a side-by-side comparison with immunoblot analyses to exclude cross-reactivity of the antibodies with other serum proteins and proteolytic fragments of fetuin-A. The assay does not cross-react with fetuin-B. The intra-assay coefficient of variation is 7.7% and the inter-assay coefficient of variation is 8.1%. The assay range is from 0.05 to 3.5 g/l.

Other laboratory tests and participant characteristics

Calcium and phosphorus were measured using a Vitros 950IRC instrument. The measurement ranges of the instrument are 0.25–3.5 mmol/l for calcium and 0.10–4.2 mmol/l for phosphorus. The coefficients of variation are 2% for calcium and 3.5% for phosphorus.

High sensitivity C-reactive protein was measured using the Roche Integra assay in 229 participants and (due to a change at the lab) the Beckman Extended Range assay in the remaining 756 participants [15]. This assay is highly correlated with the Roche Integra assay (Spearman rank correlation = 0.95 in a sample of 185 Heart and Soul Study
participants on whom we performed both assays). The inter-assay coefficient of variation was 6.7% and the intra-assay coefficient of variation was 6.2%.

Fasting serum samples were used to measure for serum total cholesterol, high-density lipoprotein (HDL) cholesterol and triglyceride levels. Low-density lipoprotein (LDL) cholesterol concentrations were estimated from the Friedewald equation [20]. Self-reported age, medical history, tobacco and alcohol use were determined from the questionnaire. Participants were instructed to bring their medication bottles to the study appointment, and research personnel recorded all the current medications.

Statistical analysis

We categorized participants into CrCl and qGFR groups by the Kidney Disease Outcomes Quality Initiative (K/DOQI) guidelines for stages I and II CKD [21]. We subdivided K/DOQI stage III CKD into two groups (45 to 59, and <45.0 ml/min/1.73 m²) as these subgroups have been shown to have a substantially different risk of mortality and other morbidity in community-based cohorts [3,22]. We had relatively few participants with CrCl <30 ml/min/1.73 m² (n = 24). Therefore, K/DOQI stages IV and V were not evaluated separately. As age-, sex- and race-specific ranges for serum cystatin-C have not been established, we categorized cystatin-C into quartiles. Albumin-to-creatinine ratio was categorized as ≤30, 31–300 and >300 mg albumin/g creatinine. Each kidney function parameter served as the independent variable and serum fetuin-A concentration served as the dependent variable. Differences in baseline characteristics were compared using analysis of variance (ANOVA) or the Kruskal–Wallis test for continuous variables and the chi-squared test for categorical variables. We conducted bivariate and multivariate linear regression to evaluate the association of each kidney function parameter with serum fetuin-A concentrations. Adjustment variables included age, sex, race, diabetes mellitus, hypertension, and serum concentrations of albumin, haemoglobin and C-reactive protein. Additionally, we used multivariate logistic regression analysis to evaluate the association between each parameter of kidney function and low serum fetuin-A (<0.55 g/l). This cut-point was chosen as it represented the lowest quartile in our cohort, and was previously associated with excess cardiovascular and all-cause mortality in persons on dialysis [14].

Previous research had found mean fetuin-A levels of 0.72 g/l (SD 0.19 g/l) among healthy controls [14]. Assuming that this population mean would apply to our study population, and using the cut-point of serum fetuin-A concentration <0.55 g/l as a clinically meaningful difference from the mean, we calculated that we had more than 99% power to detect a difference of this magnitude or larger between high and low CrCl groups (two-sided alpha = 0.05).

We also evaluated the associations of serum calcium and phosphorous concentrations with serum fetuin-A. Serum calcium was adjusted for serum albumin according to an equation commonly used in the general population: adjusted calcium (mg/dl) = measured calcium (mg/dl) + [(4.0 – serum albumin in g/dl) × 0.8]. Results were then converted to multivariate mole per litre. Participants were categorized into quartile groups of adjusted calcium (quartile I, <2.31 mmol/l; quartile II, 2.31–2.40 mmol/l; quartile III, 2.41–2.48 mmol/l; quartile IV, >2.48 mmol/l) and phosphorus (quartile I, <1.07 mmol/l; quartile II, 1.07–1.20 mmol/l; quartile III, 1.21–1.29 mmol/l and quartile IV, >1.29 mmol/l). These latter analyses were conducted with and without adjustment for CrCl.

Two-tailed P-values <0.05 were considered statistically significant. Analyses were performed using Stata Statistical Software, version 9 (College Station, TX).

Results

The mean age of the 970 study participants was 67 years, 82% were male, 40% were non-white, 71% had hypertension and 26% had diabetes mellitus. Fetuin-A concentrations were normally distributed within the study sample (Figure 1). Mean CrCl was 80 ± 29 ml/min/1.73 m², mean qGFR was 86 ± 24 ml/min/1.73 m², median albumin-to-creatinine ratio was
10 mg/g, 25–75%, range 6–20 mg/g. Baseline characteristics categorized by CrCl groups are depicted in Table 1.

### Associations of kidney function parameters with serum fetuin-A

CrCl was directly correlated with fetuin-A concentrations in bivariate analysis ($P = 0.001$). However, this association was attenuated and no longer statistically significant with adjustment for age ($P = 0.16$), and was further attenuated after multivariable adjustment (Figure 2A). Results were similar when qGFR was used as the independent variable. We observed a direct correlation between qGFR groups and fetuin-A in bivariate analysis ($P = 0.01$) that was attenuated with adjustment for age ($P = 0.71$), and further attenuated in multivariable models (Figure 2B). In unadjusted analysis, serum cystatin-C was not associated with serum fetuin-A ($P = 0.74$). With multivariable adjustment, we observed a statistically significant and graded association between serum cystatin-C and fetuin-A (with the highest fetuin-A concentrations observed among those with worse kidney function) (Figure 2C).

While the association was statistically significant, it was modest in degree, differing by only 10% from high to low quartiles. Covariates that unmasked the cystatin–C fetuin-A association included age, sex, diabetes mellitus, serum albumin and haemoglobin. The albumin-to-creatinine ratio was not associated with serum fetuin-A in either unadjusted or adjusted analysis (Figure 2D).

When we examined the likelihood of ‘hypofetuinaemia’ (serum concentration <0.55 g/l), we found a reduced odds of low serum fetuin-A among participants in the two highest cystatin-C quartiles (adjusted OR 0.52; 95% CI 0.32–0.85 and OR 0.55; 95% CI 0.33–0.91, respectively, compared with the lowest cystatin-C quartile). No significant associations were observed among CrCl (adjusted $P$ for trend = 0.72), qGFR (adjusted $P$ for trend = 0.09), or albumin to creatinine ratio (adjusted $P$ for trend = 0.31) categories and low serum fetuin-A.

### Associations of serum calcium and phosphorus with serum fetuin-A

In unadjusted analysis, we found that participants with higher serum calcium concentrations had significantly
higher mean serum fetuin-A concentrations. This association was essentially unchanged after adjusting for CrCl. Similarly, participants with higher serum phosphorous concentrations also had significantly higher mean fetuin-A concentrations in unadjusted and adjusted analyses (Figure 3).

Discussion

In the present study, we evaluated the associations of several parameters of kidney function with serum concentrations of fetuin-A within a cohort of community-dwelling persons with coronary artery disease and across a broad range of kidney function. Based on prior research [14], we hypothesized that the presence of CKD would be associated with lower serum fetuin-A concentrations and that levels would be intermediate between values observed in healthy populations and in ESRD populations. However, our results did not demonstrate an inverse association between serum fetuin-A and CrCl, quadratic GFR, cystatin-C or albumin-to-creatinine ratio—four distinct methods of evaluating kidney function, each with specific strengths and limitations.

Among persons with ESRD, vascular calcification has emerged as a powerful and potentially modifiable risk factor of all-cause mortality [23,24]. Pathological studies have shown that persons with ESRD experience accelerated atherosclerosis (involving the vascular intima), and also high rates of vascular medial calcification (Monckeberg’s sclerosis) [8,25]. Calcified vascular media may lead to decreased vascular compliance, subsequently resulting in increased left ventricular afterload and left ventricular hypertrophy [26]. Elevated systolic blood pressure, pulse pressure and left ventricular hypertrophy have been identified as independent risk factors for mortality in cohorts with ESRD [27,28] as well as in the general population [29,30].
Ketteler and colleagues [14] demonstrated that lower fetuin-A concentrations were independently associated with cardiovascular and all-cause mortality in dialysis patients. These results were recently confirmed in incident haemodialysis patients, as well as in peritoneal dialysis patients [31,32]. As fetuin-A is a potent inhibitor of vascular calcification [13,33] and as low levels had been associated with coronary artery and valvular calcification [32,34], the hypothesis was generated that the excess mortality risk observed in these studies may have been due to accelerated cardiovascular calcification among ESRD patients with lower serum concentrations of fetuin-A.

In contrast, Mehrotra and colleagues [35] studied patients with stages III and IV diabetic nephropathy (mean MDRD estimated GFR 30 ml/min/1.73 m$^2$) and significant proteinuria (mean 7 g/day). They observed higher mean serum concentrations of fetuin-A among patients with advanced diabetic nephropathy as compared with persons with diabetes but without kidney disease. Whether these findings are generalizable to persons without diabetes is unknown, particularly as fetuin-A interacts with insulin receptors and leads to insulin resistance in experimental animals [36]. Our results are consistent with those of Mehrotra et al. [35], extending the observations to persons with milder CKD and to persons without diabetes.

In unadjusted analysis, there was no association between serum cystatin-C and fetuin-A. However, after multivariable adjustment, we found a modest but statistically significant direct association between cystatin-C and fetuin-A. Thus, the observed association was in the opposite direction to that which we had hypothesized. Previous studies have suggested that cystatin-C may be a more accurate indicator of GFR than serum creatinine or its associated estimating equations [37–40]. Therefore, it is possible that concentrations of fetuin-A may increase as true GFR declines, and that this association was not observed with measured CrCl or qGFR because they may be less accurate indicators of kidney function. Alternatively, the association might be related to residual confounding. Higher cystatin-C [41] and lower fetuin-A concentrations [42] have been associated with laboratory proxies of inflammation. However, given the direct association between fetuin-A and cystatin-C, and the previously reported inverse association of fetuin-A with inflammatory biomarkers, the direction of the observed association between fetuin-A and cystatin-C would argue against confounding by inflammation. Moreover, serum albumin and C-reactive protein were not dominant confounders of the observed association between cystatin-C and fetuin-A.

We found that higher serum fetuin-A concentrations were associated with higher serum calcium and phosphorous concentrations, independent of kidney function—a finding of potential interest given the role of fetuin-A as an inhibitor of calcification. Osawa and colleagues [43] reported no association between serum fetuin-A concentrations and total and albumin-corrected calcium, and an inverse correlation with serum phosphorous concentrations among healthy Japanese volunteers without kidney disease. Several possibilities may explain the differences in our results. First, our subjects were older and ethnically diverse. Second, a different assay for fetuin-A was used, although inter- and intra-assay coefficients of variation are relatively low for both assays. Lastly, all participants in our study had coronary artery disease and many had mild to moderate kidney disease. Structure-function studies have suggested that fetuin-A solubilizes calcium phosphate crystals by direct and reversible binding, reminiscent of the process in which apolipoproteins solubilize lipids in solution [33]. In experimental mice with kidney disease that were provided high phosphate feeding, wild-type mice developed high serum calcium and phosphorous concentrations, but did not develop extraosseous calcification. In contrast, fetuin-A knockout mice developed significant soft-tissue calcification while maintaining normal serum calcium and phosphorous concentrations [44]. These findings suggest that under certain conditions, elevated serum concentrations of calcium and phosphorous may reflect improved ion solubility [45]. In our study population, the presence of pre-existing cardiovascular disease and alterations in mineral metabolism conferred by CKD may have led to the up-regulation of fetuin-A in association with elevated concentrations of calcium and phosphorous, perhaps to offset the propensity towards dystrophic mineralization. A better understanding of the regulation of fetuin-A in the presence and absence of uraemia and vascular disease and at varying concentrations of calcium and phosphorous is required.

A relative strength of our study is the availability of several parameters of kidney function and measurement of multiple potential confounding variables. However, there are several limitations that should be considered when interpreting our results. Our study could not evaluate the association of advanced CKD (stages IV and V) and serum fetuin-A. Whether a threshold of kidney function exists, at which fetuin-A concentrations decline, cannot be addressed in our study sample. Future studies evaluating whether fetuin-A concentrations are decreased among persons with stages IV and V CKD before the initiation of dialysis are warranted particularly as such persons are known to have high prevalence of vascular calcification [46] and because such an approach would advance our understanding of the relative contribution of uraemia vs the effects of dialysis therapy itself on fetuin-A concentrations. The cross-sectional design of our study does not allow for causal inference or evaluation of direction of associations. Finally, our study participants were mostly male, and all had coronary artery disease. Our results may therefore not be generalizable to women or to persons without coronary artery disease.

In summary, we found no evidence that mild-to-moderate CKD was associated with lower concentrations of serum fetuin-A in a well-characterized cohort of 970 persons with coronary artery disease.
Serum fetuin-A was associated with higher serum calcium and phosphorous concentrations, independent of kidney function. The regulation of serum fetuin-A warrants further study. For now, the mechanisms explaining the association between CKD and vascular calcification remain elusive. Since hypofetuinemia appears to be uniquely associated with advanced kidney disease, uremia and/or dialysis may be directly involved in fetuin-A regulation.

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Conflict of interest statement. None declared.

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