

ORIGINAL ARTICLE

Subclinical atherosclerosis and fetuin-A plasma levels in essential hypertensive patients

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The intima-media thickness (IMT) is considered as a surrogate marker for atherosclerotic disease. The aim of this study was to analyze the relationship of carotid IMT with fetuin-A in patients with essential hypertension (EH) and normal renal function. The plasma levels of fetuin-A, interleukin 6 (IL-6), tumor necrosis factor- α (TNF- α) and the biomarker of oxidative stress 8-iso-PGF2alpha were assayed in samples from 105 untreated EH patients. Carotid IMT measurements were also performed. EH was studied overall and after dividing in EH with IMT \geq and $<$ 0.9 mm. All of the biomarkers were significantly different between the two subgroups, in particular, the fetuin-A level was lower in the patients with an IMT \geq 0.9 mm. In the overall group, the linear analysis of correlation demonstrated that the IMT was significantly inversely correlated with the fetuin-A level ($r = -0.40$, $P < 0.0001$) and directly with TNF- α ($r = 0.39$, $P < 0.0001$), IL-6 ($r = 0.38$, $P < 0.0001$) and 8-iso-PGF2alpha ($r = 0.356$, $P < 0.0003$). The multiple regression analysis performed that assigned IMT as a dependent variable showed that fetuin-A ($\beta = -0.268$, $P < 0.0001$) was independently correlated with the IMT. Receiver-operator curves demonstrated that fetuin-A levels have a predictive power of IMT $>$ 0.9 mm (AUC (area under the curve) 0.738, $P < 0.0001$). Our results suggest that in EH, fetuin-A is associated with the IMT independently of oxidative stress and renal function, thus predicting increases in the IMT.

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INTRODUCTION

Subclinical organ damage represents an intermediate stage in the continuum of vascular disease and is a determinant of the overall cardiovascular risk. Arterial stiffness represents an independent predictor for all causes of cardiovascular mortality and morbidity in patients with essential hypertension (EH), diabetes or end-stage renal disease.^{1,2} Ultrasound measurements of the intima-media thickness (IMT) are widely used in clinical studies as a surrogate marker for atherosclerotic disease.^{3,4} The so-called response-to-injury hypothesis for atherosclerosis states that the initial damage involves the endothelium, with increased oxidative stress leading to endothelial activation and dysfunction. Moreover, atherosclerosis is considered to be an inflammatory disease.⁵

Fetuin-A is a member of the cystatin superfamily of cysteine protease inhibitors. It is a negative acute-phase glycoprotein produced by the liver and is detectable in the serum. Fetuin-A is an endogenous inhibitor of the insulin receptor tyrosine kinase, having an unclarified role in diabetes mellitus. Recently, a cross-sectional study demonstrated that lower fetuin-A levels were associated with macrovascular complications in high-risk type 2 diabetes patients⁶ and with the severity of atherosclerosis in patients with peripheral vascular disease

and normal renal function.⁷ Fetuin-A is reported to be a systemically acting inhibitor of extraosseous calcification in fetuin-A knockout mice.^{8,9}

Several studies reported that lower fetuin-A serum levels were associated with mortality and cardiovascular events in cohorts with end-stage renal disease, whereas a population-based study linked high plasma fetuin-A levels to an increased risk of myocardial infarction and ischemic stroke.¹⁰ Diabetic patients having carotid plaque were reported to be characterized by low fetuin-A levels,¹¹ yet only limited data are available in patients with normal kidney functions or moderate renal impairment and advanced atherosclerosis.^{10,12}

The aim of this cross-sectional, observational study was to analyze the relationship of subclinical atherosclerosis, as evaluated by carotid IMT measurements with fetuin-A, and certain biomarkers of both oxidative stress and inflammation in patients having EH and normal renal function who are free from cardiovascular disease.

METHODS

In accordance with the Declaration of Helsinki and institutional guidelines, the protocol for this study was approved by the local Ethics Committee, and the

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subjects were aware of the investigational nature of the study and agreed to participate after giving informed consent.

Study population

We enrolled 105 consecutive EH patients. These patients visited our Nephrology and Hypertension Unit for the management and/or the differential diagnosis of their hypertensive disease. All of the patients were untreated and exhibited normal renal function according to the NKF guidelines.¹³

Fifty-five healthy, age-matched individuals among our staff were enrolled as the control group. Both the control individuals and hypertensive patients were Caucasian. The glomerular filtration rate was estimated (eGFR) using the Chronic Kidney Disease Epidemiology Collaboration (CKD-EPI) equation.¹⁴ Before the inclusion in the study, all of the subjects underwent a routine chemical analysis, including a hemochrome with a white blood cell count transaminase, gamma glutamyltransferase and alkaline phosphatase evaluations.

Patients were defined as hypertensive when their clinical blood pressure was >140/90 mm Hg, and the severity of hypertension was defined according to the ESH guidelines of 2007.¹⁵ The clinical blood pressure was calculated as the average of three consecutive measurements using an automatic sphygmomanometer after the subject had been sitting for 5 min. Secondary or complicated forms of hypertension were excluded by a clinical examination and the determination of the serum creatinine, serum and urinary electrolytes, plasma catecholamine levels and renin activity and renal echography.

The exclusion criteria were as follows: age younger than 18 years or older than 70 years; secondary or accelerated-malignant arterial hypertension; a history of transitory ischemic attack or stroke; a history of angina, coronary heart disease or myocardial infarction, heart failure or abnormalities of cardiac rhythm or conduction under pharmacological treatment. Patients with diabetes or acute infection and subjects having liver disease were also excluded from the study. No patients were on statin treatment.

Study protocol

At 0900 hours on the day of the study, the overnight-fasted patients were placed in a supine position, and blood samples were obtained from an indwelling forearm venous catheter to assay the serum creatinine, lipid profile, fetuin-A, interleukin 6 (IL-6), tumor necrosis factor alpha (TNF- α), as well as isoprostane 8-iso-PGF2alpha as a biomarker of oxidative stress. The basal blood pressure was also measured. All of the patients underwent an echo-color Doppler of the carotid arteries to measure the IMT.

Laboratory methods

The serum creatinine was measured using the Jaffé method (Instrumentation Laboratory Company, Lexington, MA, USA). The plasma total cholesterol (TC), triglycerides (TG) and high-density lipoprotein-cholesterol (HDL-C), after phosphotungstic acid precipitation, were measured using standard enzymatic colorimetric procedures (Roche Diagnostics, Basel, Switzerland) using a COBAS MIRA plus autoanalyzer (Roche Diagnostics). The low-density lipoprotein-cholesterol (LDL-C) was calculated by the Friedewald formula: $LDL-C (mg dl^{-1}) = TC (mg dl^{-1}) - TG (mg dl^{-1})/5 - HDL-C (mg dl^{-1})$.

The levels of fetuin-A, TNF- α , IL-6 and 8-iso-PGF2alpha were measured using a solid-phase-specific sandwich enzyme-linked immunosorbent assay. Standard curves were constructed using the appropriate concentration for each factor. Fetuin-A was measured using a commercial kit (Epitope Diagnostics, San Diego, CA, USA); the sensitivity was 2.5 ng ml⁻¹.

TNF- α was assayed using an Amersham Biosciences kit (Little Chalfont, England). The sensitivity was <5 pg ml⁻¹, and the reproducibility of the intra- and between-assays had a coefficient of variation <10%. IL-6 was assayed using a commercially available enzyme-linked immunosorbent assay kit (Pierce Biotechnology Rockford, IL, USA). The sensitivity, inter-assay and intra-assay coefficients of variation were <1 ng l⁻¹, <10% and <10%. Isoprostane 8-iso-PGF2alpha was analyzed using a commercial kit (Assay Design, Ann Arbor, MI, USA). The sensitivity was 16.3 pg ml⁻¹, and the inter-assay coefficient of variation was less than 9%.

Assessment of the carotid IMT

Carotid ultrasonography to assess the IMT was performed by two investigators trained for this purpose before starting the study. A Logic P5 PRO (General Electric Company, Milan, Italy) ultrasound device paired with a 5–10 MHz multifrequency high-resolution linear transducer was used to perform the measurements of the IMT. The measurements were performed on the common carotid artery after the examination of a longitudinal section of 10 mm at a distance of 1 cm from the bifurcation. The measurements were performed in the proximal wall and in the distal wall in the lateral, anterior and posterior projections by following an axis that was perpendicular to the artery to discriminate two lines: one for the intima–blood interface and the other for the media–adventitia interface. A total of six measurements were obtained for the right carotid artery and six for the left carotid using the average values (average IMT) calculated automatically by the software. The measurements were obtained with the subject lying down, with the head extended and slightly turned opposite to the carotid being examined, following the recommendations of the Mannheim Carotid Intima-Media Thickness Consensus.¹⁶ The average IMT was considered abnormal when >0.90 mm or when there were atherosclerotic plaques with a diameter of 1.5 mm or a focal increase of 0.5 mm or 50% of the adjacent IMT.¹⁶

Statistical analyses

The results are provided as the mean \pm s.d. The differences between the groups were evaluated using an ANOVA and the Student's *t*-test. In the overall group, the univariate associations between the variables were assessed using the Pearson correlation coefficients. Stepwise multiple linear regression analyses were used to test the independent correlations of the average IMT. Initially, the age, sex ($M = 1$; $F = 0$), clinical (or 24 h) systolic and diastolic blood pressures, serum glucose, smoking habits, uric acid, body mass index, eGFR and CKD-EPI, LDL and HDL cholesterol levels were included in the models. Subsequently, we ran the models again, adding the fetuin-A, TNF- α , IL-6 and 8-iso-PGF2alpha levels after the exclusion of the parameters not associated with the IMT in the preliminary analyses.

Receiver-operator curves (ROCs) were built to assess the power of fetuin-A to predict an IMT >0.9 mm in the overall group. The curves were built using the normal transformed variables of biomarkers with a normal signal-to-noise model. The biomarker values were ranked in deciles, to obtain 10 points to build the ROCs. The ROC areas and D-prime values were considered to be estimators of the predictive power. The statistical analyses were performed using the SYSTAT DATA software package, version 5.2 (Systat, Evanston, IL, USA) and MedCalc software (Mariakerke, Belgium).

RESULTS

Table 1 gives the demographic and clinical data for the 105 hypertensive patients and 55 control individuals. None of the patients exhibited significant hemodynamic carotid stenosis (>50%). The mean plasma levels of fetuin-A, 8-iso-PGF2alpha and of the other biomarkers of inflammation observed in the hypertensive subgroups with IMTs \geq and <0.9 mm are shown in Table 2. All of the biomarkers were significantly different between the two subgroups, in particular, the fetuin-A level was lower in patients with an IMT \geq 0.9 mm.

In the overall group, the linear analysis of correlation demonstrated that the IMT was significantly inversely correlated with fetuin-A ($r = -0.40$, $P < 0.0001$; Figure 1) and directly correlated with TNF- α ($r = 0.39$, $P < 0.0001$), IL-6 ($r = 0.38$, $P < 0.0001$) and 8-iso-PGF2alpha ($r = 0.356$, $P < 0.0003$).

Moreover, fetuin-A correlated significantly and inversely with TNF- α ($r = -0.75$, $P < 0.0001$), IL-6 ($r = -0.5959$, $P < 0.0001$) and 8-iso-PGF2alpha ($r = -0.72$, $P < 0.0001$). No significant correlations between fetuin A with glycemia or the eGFR ($r = 0.04$ and 0.06, respectively) were found.

A trend of an association between fetuin-A and age was observed, but it was not statistically significant ($r = -0.169$; $P = 0.08$). The

Table 1 Demographic and clinical data of 105 essential hypertensives and 55 controls

	Controls (M/W 32/23)	EH (M/W 69/36)	P
	mean ± s.d.	mean ± s.d.	
Age (years)	45 ± 10	46.9 ± 11.8	0.31
BMI (kg m ⁻²)	25.6 ± 2.8	28.3 ± 4.6	0.0001
Smoke	25/55	48/105	0.897
Systolic blood pressure (mm Hg)	117 ± 6	148 ± 7.5	0.0001
Diastolic blood pressure (mm Hg)	73 ± 4	95 ± 4.5	0.0001
Serum glucose (mg dl ⁻¹)	89 ± 10	100 ± 12	0.0001
Serum creatinine (mg dl ⁻¹)	0.91 ± 0.09	0.9 ± 0.1	0.535
CKD-EPI (ml min ⁻¹ per 1.73 m ²)	98 ± 12	95.5 ± 9	0.140
Serum uric acid (mg dl ⁻¹)	4.8 ± 0.3	5.1 ± 0.91	0.019
Serum cholesterol (mg dl ⁻¹)	201 ± 12	204 ± 19	0.289
HDL serum CH (mg dl ⁻¹)	44 ± 8	42 ± 10	0.201
LDL serum CH (mg dl ⁻¹)	125 ± 28	133 ± 40.9	0.188
Serum triglyceride (mg dl ⁻¹)	132 ± 14	129 ± 32	0.509
Fetuin-A (g l ⁻¹)	0.95 ± 0.14	0.8 ± 0.12	0.0001
TNF-α (ng ml ⁻¹)	1.35 ± 0.45	3.3 ± 0.61	0.0001
IL-6 (ng ml ⁻¹)	31.9 ± 6.25	67.16 ± 20.2	0.0001
8-iso-PGF2α	77.7 ± 10	200.14 ± 87	0.0001
Intima-media thickness (mm)	0.76 ± 0.19	0.91 ± 0.34	0.0001

Abbreviations: BMI, body mass index; CH, cholesterol; CKD-EPI, Chronic Kidney Disease Epidemiology Collaboration; HDL, high-density lipoprotein; IL, interleukin; LDL, low-density lipoprotein; TNF-α, tumor necrosis factor-α.

Table 2 Plasma levels of fetuin-A, TNF-α, IL-6, 8-iso-PGF_{2α} in the subgroups of EHs having IMT <0.9 mm and IMT >0.9 mm

	IMT <0.9 mm	IMT >0.9 mm	P
	n = 52	n = 53	
Fetuin-A (g l ⁻¹)	0.85 ± 0.1	0.76 ± 0.04	0.0001
TNF-α (ng ml ⁻¹)	3.1 ± 0.5	3.5 ± 0.0.6	0.0001
IL-6 (ng ml ⁻¹)	55.5 ± 32.6	79.5 ± 43	0.003
8-iso-PGF2α	171 ± 81	230 ± 82	0.0001

Abbreviations: EH, essential hypertensive; IL, interleukin; IMT, intima-media thickness; TNF-α, tumor necrosis factor-α; 8-iso-PGF_{2α}, 8-Iso-Prostaglandin F_{2α}.

average IMT was strongly associated with age ($r = 0.46$; $P < 0.001$). Moreover, after an adjustment for age, the average IMT was significantly higher in men than in women (0.937 ± 0.24 vs. 0.799 ± 0.22 mm; $P = 0.002$).

In the multiple regression analysis considering IMT as a dependent variable in a model comprising age, sex ($M = 1$, $F = 0$), clinical systolic and diastolic blood pressures and serum glucose, uric acid, body mass index, eGFR and CKD-EPI, LDL and HDL cholesterol levels, only the age ($\beta = 0.482$; $P < 0.001$) and gender ($\beta = 0.257$;

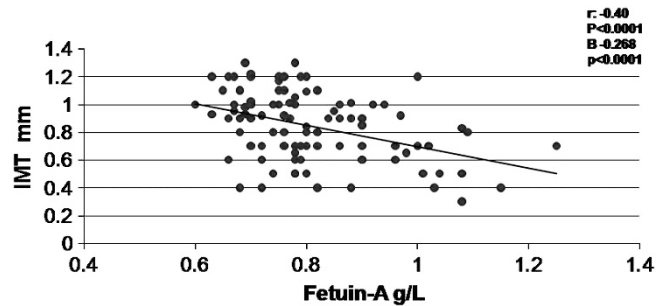


Figure 1 Negative correlation between plasma levels of fetuin-A and intima-media thickness (IMT). A full color version of this figure is available at the *Hypertension Research* journal online.

$P = 0.004$) of the patients were associated with the IMT. Similar results were obtained when the clinical BP values were replaced by the corresponding 24-h readings in the multiple regression model. When the multivariate regression analysis was reperformed, including fetuin-A, TNF-α, IL-6 and 8-iso-PGF_{2α}, along with age and sex, in the new model, fetuin-A remained significantly associated with the IMT (Table 3). The ROCs demonstrated that the fetuin-A levels have with a predictive power for an IMT >0.9 mm (AUC (area under the curve) 0.738, $P < 0.0001$): fetuin-A values of 0.77 g l^{-1} predict an IMT >0.9 mm with a 70% sensitivity and a 68% specificity (Figure 2).

DISCUSSION

This study demonstrates that in essential hypertensive patients having a normal eGFR, lower fetuin-A levels appear to be associated with IMT, a surrogate marker of an atherosclerotic burden. Indeed, fetuin-A correlates inversely with the IMT and has a predictive power for the IMT.

Fetuin-A complexes with calcium and phosphorus in the circulation and prevents the precipitation of these minerals in the serum.¹⁷ Fetuin-A is regarded as a marker for vascular inflammation and is one of the most potent negative regulators of vascular ossification-calcification.^{18,19} In fetuin-A-deficient mice, the aorta was found to be devoid of calcification and fibrosis, whereas the peripheral vessels in the skin and kidney showed evidence of extensive calcification; furthermore, the small artery involvement preceded the impairment of renal function.^{17,18,20}

The data presented herein support the relationship between fetuin-A and IMT, suggesting that decreases in the fetuin-A level could have a role in the development of the vascular changes that are peculiar to hypertensive patients; this role appears to be independent of other confounding factors, such as renal function. Nevertheless the results of this study are not an unambiguous demonstration for the causative role of fetuin-A. The functions and regulatory mechanisms of fetuin-A remain to be fully elucidated and seem to differ according to the pathophysiological characteristics of the population studied.^{5,9,10,21} Caglar *et al.*²¹ showed that the fetuin-A levels significantly increased after renal transplantation, that is, after a nearly complete restoration of the renal function. Recently, the same authors²² hypothesized that fetuin-A may be one of the contributing factors for the development of endothelial dysfunction in CKD patients. It is well known that the latter represents the initial step in the development of atherosclerosis, which is recognized as an inflammatory disease.⁶

It is noteworthy that in the present study, fetuin-A correlated significantly and inversely with IL-6. We previously demonstrated that in chronic kidney disease, even when not severe, the inflammatory

Table 3 Variables independently associated with average IMT in multiple regression analysis (adjusted squared multiple $R=0.328$)

Effect	Regression coefficient		Standardized regression		t	P-value
	(β)	s.e.	coefficient (β)	Tolerance		
Constant	0.705	0.177	0.000	0.000	3.979	0.000
Gender	0.114	0.042	0.233	0.927	2.738	0.007
Age	0.009	0.002	0.438	0.908	5.092	0.000
Fetuin-A	-0.494	0.157	-0.268	0.919	-3.139	0.002

Abbreviation: IMT, intima-media thickness.

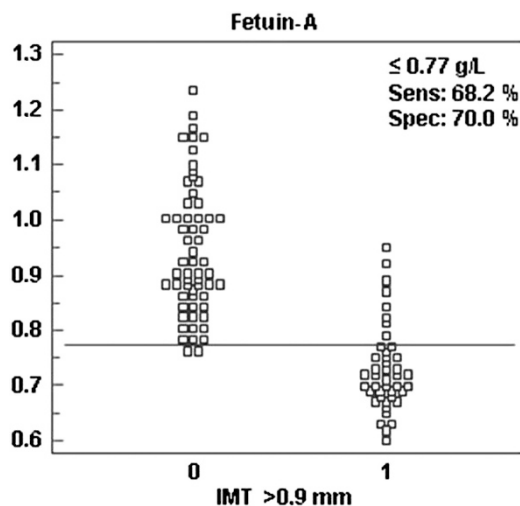


Figure 2 Dot plot distribution of fetuin-A values according to the presence of intima-media thickness (IMT) ≥ 0.9 mm by plasma levels in 105 essential hypertension patients. A full color version of this figure is available at the *Hypertension Research* journal online.

processes assayed by the IL-6 plasma levels are increased and linked to endothelial dysfunction, which progressively worsens with the decline of renal function.²³ Furthermore, IL-6 emerged as the strongest independent predictor in a study aimed at evaluating the relationship of some biomarkers of inflammation with the burden of coronary artery disease, as detected by coronary angiography.²⁴

In this study, fetuin-A correlated with the IMT, even independently of the lipid profile and oxidative stress. In fact, considering the well-known relationship between atherosclerosis and oxidative stress, one could expect that its biomarker, 8-iso-PGF $_{2\alpha}$, should correlate with the IMT. However, the significant linear correlation was removed by multiple analyses. These observations corroborate the hypothesis of the role of inflammation biomarkers in vascular changes and suggest that in patients at risk for atherosclerotic disease, the fetuin-A level is decreased even in the early stage of vascular disease, before plaque formation and independently of renal dysfunction.

This hypothesis is in agreement with the data by Emoto¹¹ who demonstrated in 416 patients with type 2 diabetes mellitus and without renal dysfunction that the circulating fetuin-A levels were significantly lower in patients with atherosclerotic calcified plaques than in those without.⁷ Furthermore, the fetuin-A levels were inversely associated with the presence of calcified plaques, and the authors concluded that fetuin-A may inhibit the calcification of

atherosclerotic plaques independently of the dialyzed condition.^{25,26} Nevertheless, recent data from the Rancho Bernardo Study²⁷ showed that lower fetuin-A levels were independently associated with coronary artery calcification but not with the IMT in community-living individuals free of cardiovascular disease. These results led to the hypothesis that fetuin-A could be associated with subclinical coronary atherosclerosis but not with atherosclerosis of other vascular beds, thus raising the question of whether it is possible that a circulating protein could target a specific vascular bed. Indeed, experimental studies showed a linkage between fetuin-A and several vascular beds.¹⁷

This study has some limitations and strengths. The major limitations consist of the small group of subjects and by the characteristics of a cross-sectional study. The strengths include the well-characterized population, the homogeneous population ethnicity and the absence of both previous cardiovascular events and pharmacological treatment.

In conclusion, our results suggest that in EH, fetuin-A is associated with the IMT independently of oxidative stress and renal function and is a predictor of increases in the IMT. Larger studies are required to confirm these observations.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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