Published in final edited form as:
*J Surg Res*. 2010 September ; 163(1): 12–17. doi:10.1016/j.jss.2010.04.010.

# VALIDATION OF PLASMA BIOMARKERS IN DEGENERATIVE CALCIFIC AORTIC STENOSIS

**Giovanni Ferrari, PhD**[1], **Rachana Sainger, PhD**[1], **Erik Beckmann, MD**[1], **Gianluca Keller**[1], **Pey-Jen Yu, MD**[2], **Maria Cristina Monti, PhD**[3], **Aubrey C. Galloway, MD**[2], **Richard L. Weiss**[1], **William Vernick**[1], and **Juan B. Grau, MD**[1]

[1]University of Pennsylvania School of Medicine, Department of Surgery, Division of Cardiovascular Surgery, Philadelphia, PA

[2]New York University School of Medicine, Department of Cardiothoracic Surgery, New York, NY

[3]Columbia University, Department of Biostatistics, New York, NY

## Abstract

**Introduction—**Calcific aortic stenosis (CAS) is the most common acquired valvular disorder in industrialized countries. This study investigates the correlation of different known biomarkers for CAS as a first step towards the development of a panel of biomarkers that can be used in prognostic staging.

**Methods—**Venous blood samples were obtained from both patients with CAS scheduled for surgery and healthy individuals. Plasma levels of fetuin-A, NT-proBNP, BNP, Homocysteine and Osteopontin were measured by ELISA. CAS was measured by echocardiography and was defined as an aortic valve area of less than 2.0 $cm^2$. Non-paired t tests were used for comparison.

**Results—**CAS was present in 33 subjects (mean age 75.9 years) and absent in 11 subjects (mean age 55.36 years). Individuals with CAS exhibited higher plasma levels of NT-proBNP (1.33 vs 0.73 pmol/ml, $p<0.05$), BNP fragment (1.47 vs 0.34 ng/ml $p<0.05$) and Osteopontin (60.79 vs 25.42 ng/ml $p<0.05$) compared to controls. Fetuin-A levels were lower in individuals with CAS than in healthy controls (0.25 vs 0.34g/l, $p<0.05$). Asymmetric dimethylarginine (ADMA) were lower (1.08 vs 1.1 μmol/l, $p>0.05$) while homocysteine levels (20.34±2.14 Vs 19.23±4.19 $p>0.05$) were higher in the CAS patients.

**Discussion—**This study demonstrates a direct correlation of NT-pro-BNP, BNP and Osteopontin and the presence of CAS while Fetuin A showed an inverse correlation. Plasma ADMA and homocysteine levels were comparable in the CAS patients and healthy individuals. This is the first study in which several biomarkers previously studied independently in patients with CAS have been investigated simultaneously in the same study population.



To whom correspondence should be addressed: Giovanni Ferrari, PhD, Research Assistant Professor of Surgery, University of Pennsylvania School of Medicine, Harrison Department of Surgical Research, 500 S. Ridgeway Ave, Room 257, Glenolden, PA 19036, Phone : (267) 350 9617, Fax : (267) 250 9627, Giovanni.Ferrari@uphs.upenn.edu.

**Keywords**

Calcific Aortic Stenosis (CAS); Biomarkers; Heart diseases; Aortic Valve Calcification; Prevention; Fetuin A; NT-proBNP; Brain Natriuretic Peptide (BNP); Osteopontin (OPN); Asymmetric dimethylarginine (ADMA); Homocysteine

## Introduction

Calcific aortic stenosis (CAS) is a slow but progressive pathological condition of the aortic valve (1,2). With an estimated prevalence of 5.2 million affected people in the United States, CAS represents the most common type of valvular disease (3). Symptomatic aortic stenosis (AS) is associated with poor prognosis, and the current treatment of choice is surgical valve replacement (4), either with mechanical or biological prostheses. Other treatment options are aortic valvuloplasty or percutaneous valve replacement. Balloon aortic valvuloplasty is a well-established and well-studied procedure with nontrivial complication rates, very high rates of recurrent stenosis and moderately high rates of aortic insufficiency (5). The clinical and pathomorphological presentation of CAS is quite wide-ranging. Initial phases of the disease include only mild thickening of the valve, whereas more advanced stages comprise serious impairment of leaflet motion with subsequent limitation of blood flow through the valve (1). These two medical conditions are generally known as aortic valve sclerosis (AVSc) and aortic valve stenosis (AVS), respectively.

Since there is no medical therapy available for aortic valve stenosis, surgery represents the only definitive therapy. At the present time, surgical valve replacement in any of its forms leaves the underlying mechanism that caused the original valvular degeneration, untreated. In addition, the current understanding of the pathophysiological mechanisms underlying CAS is still not fully elucidated (1,2,6). For many decades, calcific aortic stenosis has been considered an expected consequence of normal aging. The pathogenesis was viewed as a degenerative process resulting from prolonged “wear and tear” of the aortic valve with concomitant passive calcium deposition in the valve leaflets (1,2). However, recent data indicates that this concept is mostly obsolete. CAS is now thought to be an active cellular process that develops within the valve leaflets.

Mechanical stress on the aortic valve in addition to atherosclerotic risk factors, leads to valvular endothelial dysfunction/leakage, followed by deposition of lipids and other compounds. This triggers inflammation, which in turn activates valvular myofibroblasts resulting in their osteoblastic transdifferentiation. These events provide the basis for further changes involving extracellular matrix remodeling and neo-vascularization, ultimately leading to active calcification (1,2). These calcific changes primarily occur at the aortic side of the valve leaflets, which is the region with the highest turbulence suggesting mechanical stress might be one of the causes triggering calcification (1,2).

Due to the multiple biological pathways leading to CAS, a comprehensive screening of these patients using multiple biomarkers, in conjunction with genomic analysis, would provide the information necessary to optimize future therapeutic interventions. The general purposes of biomarkers include disease identification, grading disease severity, providing pathophysiological clues, prognostic information, and assessing the effects of different therapeutic interventions. The use of biomarkers and screening programs to assess the risk of future disease has helped improve outcomes across a wide a spectrum of disease states. CAS is a complex multifactorial process; therefore the analysis of the different stages of aortic valve degeneration can not be exclusively based on the quantification of a single biomarker per series. This study simultaneously correlates different published biomarkers

for CAS in a single cohort of patients with different degrees of aortic valvular stenosis and calcification. This is a first step towards the development of a battery of biomarkers that could become useful in staging patient with CAS.

## Materials and Methods

### Patients Population

Patients with any degree of CAS seen in the echocardiography laboratory undergoing routine evaluation and patients scheduled to undergo aortic valve surgery were selected. Clinical information was obtained by patient interview and chart review. Patient selection was limited to those > 65 years of age. Exclusion criteria included: patients with serum creatinine ≥ 1.5mg/dl, bicuspid aortic valve, premature menopause and/or osteoporosis, prior aortic valve surgery, rheumatic heart disease, endocarditis, active malignancy, chronic liver failure, calcium regulation disorders (hyperparathyroidism, hyperthyroidism, and hypothyroidism), and chronic or acute inflammatory states (sepsis, autoimmune disease, and inflammatory bowel disease).

### Echocardiographic and Doppler data

All patients had a comprehensive echocardiographic assessment including, M-mode, two-dimensional and color Doppler echocardiography, conducted by a certified echocardiographer using commercially available ultrasound systems. All measurements were performed according to the American Society of Echocardiography recommendations (7). The presence of aortic stenosis was defined as an aortic valve area $<2.0 cm^2$. Aortic valve calcification was assessed, and a calcium score of 1 to 4 was assigned for each patient by a single cardiologist based on the method described by Rosenhek et al. (8): 1- no calcification; 2 - mildly calcified (small isolated spots); 3- moderately calcified (multiple larger spots); 4 - severely calcified (extensive thickening and calcification of all cusps).

### Clinical and Biochemical Data

Upon obtaining patient's informed consent twenty cc of peripheral venous blood were drawn. Plasma, serum and buffy coat were obtained and stored. Half of the collected blood sample was allowed to clot at room temperature for a minimum of 30 min. It was then centrifuged for 20 min at 1,200 × g at 4°C and the supernatant was collected and sent to the in-hospital chemistry laboratory to analyze levels of calcium, phosphate, alkaline phosphatase, albumin, calcitonin, parathyroid hormone, CRP, ESR, fibrinogen, total cholesterol, low-density lipoprotein cholesterol, high-density lipoprotein cholesterol, and triglycerides. The other half of the blood sample was collected in the presence of anticoagulant EDTA, and centrifuged for 20 min at 1,200 × g at 4°C. Plasma levels of fetuin-A (Biovendor Research and Diagnostic Products, Chandlier, NC), NT-proBNP (Phoenix Pharmaceuticals, Burlingame, CA), Osteopontin (R&D Systems, Minneapolis, MN), BNP and ADMA (ALPCO Diagnostics, New Hampshire, USA) were measured by ELISA according to manufacturer's instructions. Plasma homocysteine (Diazyme Laboratories, Poway, CA), levels were estimated spectrophotmetrically. All the assays were done in triplicates. Absorbance was measured using the ELx 808 Ultra Micro plate reader (BIOTEK Instruments Inc., USA). For all the assays, results were calculated using the 4-parameter logarithm using the KC Junior software. The presented study has been conducted in accordance to the code of ethical standards of the University of Pennsylvania and New York University Schools of medicine IRB guidelines.

### Statistics

The data were analyzed using SPSS software (version 15; SPSS). Continuous variables were expressed as mean ± standard deviation. Comparisons of continuous variables between groups were performed with the Student's t test or nonparametric (Mann-Whitney U test) tests as appropriate, depending upon normal distribution. All categorical variables were compared between groups with the chi-square or Fisher's exact test. Correlations, such as the correlation between plasma biomarkers levels and aortic valve calcification scores, were characterized by Spearman's Rank Correlation Test. A value of $p < 0.05$ was considered to be significant, and a value between 0.05 and 0.10 was considered to be showing a trend toward significance.

## Results

### Baseline Patients Characteristics

On the total of 33 patients, 45 % were male. Their age was 75.9±7.17 years. The initial AVA was 0.75±0.267, 27 patients had systemic hypertension, 12 had diabetes and 4 had cholesterol level > 200mg. Table I further characterizes the study groups.

### Selection of Biomarkers

We looked at all published biomarkers for aortic stenosis and created table II, then we selected the ones with the best performance to be included in a future battery intended to better stage patients with aortic valve stenosis and tested them in our own series. We selected ADMA, fetuin-A, CaxP, Natriuretic peptides and osteopontin as the most promising candidates at the present time as they have demonstrated the best potential in the published literature up to date (Table II). In addition, we included plasma homocysteine levels, a less promising to biomarker of aortic valve stenosis, based on the results reported so far.

ADMA level correlate with the degree of AVS and is involved in endothelial cell dysfunction (6), which represents an important process in the early stages of the pathogenesis of CAS . Fetuin-A is interesting due to its *in vitro* ability to inhibit calcification (9). Natriuretic peptides seem to be very promising candidates, as there is great consistency in the literature on their ability to reflect the activity and progression, as well as to predict the prognosis of CAS. Although CaxP has only been correlated with the severity of CAS, it has been selected as high levels of calcium and phosphorus have been linked to increased calcification (10). Homocysteine is a marker associated with endothelial dysfunction, however due to the poor statistical power, the exact role of homocysteine in CAS could not be established (11,12). Osteopontin is of special interest as a biomarker for CAS, since it is the only molecule directly involved in the ectopic calcification phenomenon that occurs in the latter stages of CAS (13,14). Table II.

**Correlation among Circulating Biomarkers in Calcific Aortic Stenosis—**To determine if the selected biomarkers could be tested in the same group of patients we selected 44 individuals from our tissue-bank database (33 patients with Aortic Valve calcification score of 3 or 4 and 11 subjects with no signs of Calcific Aortic Stenosis). We correlated the degrees of CAS with the circulating levels of multiple biomarkers in the same group of patients.

After informed consent had been obtained, venous blood samples were drawn from both patients with CAS scheduled for surgery and healthy individuals. Plasma levels of fetuin-A, NT-proBNP, BNP, ADMA and Osteopontin were measured by ELISA as described. The degree of aortic valve stenosis and calcification was measured by echocardiography. Aortic valve calcification was assessed using a validated grading system based on the method

described by Rosenhek et al. (8): (1) no calcification; (2) mildly calcified (small isolated spots); (3) moderately calcified (multiple larger spots); (4) heavily calcified (extensive thickening and calcification of all cusps). There were no significant differences in the gender, age, co-morbidities, blood calcium level, and calcium-phosphorus product (CaxP) between the two groups. Aortic valve calcification score was 3.29 ± 0.73 in AS patients and 1.3 ± 0.49 in controls ($p < 0.001$).

CAS was present in 33 subjects (mean age 75.9 years) and absent in 11 subjects (mean age 55.36 years). Aortic valve calcification scores were 3 or 4 in patients with AVS and 1 in controls. Individuals with AVS exhibited significantly higher plasma levels of NT-proBNP (1.33 vs 0.73 pmol/ml, $p < 0.05$), BNP fragment (1.47 vs 0.35 ng/ml $p < 0.001$) and Osteopontin (60.79 vs 25.42 ng/ml $p < 0.05$) compared to controls. Fetuin-A levels were lower in individuals with CAS than in healthy controls (0.25 vs 0.34g/l, $p<0.05$). ADMA levels were comparable in CAS patients and controls (1.08 vs 1.10 mmol/l, $p>0.05$), as well as plasma homocysteine levels (20.34±2.14 vs 19.23±4.19, $p>0.05$) (Table III, Figure 1). This study demonstrates a direct correlation of NT-pro-BNP, BNP, Osteopontin with CAS, while Fetuin A levels showed, accordingly to the data reported in literature, an inverse correlation with the presence of CAS. ADMA and homocysteine levels are not significantly different between patients and controls.

## Discussion

Calcific aortic stenosis is a multifactorial process that underlies different types of aortic valve pathology such as: Rheumatic calcific aortic stenosis, Senile Calcific Aortic Stenosis, and Congenital Heart Diseases such Bicuspid Aortic Valve (15,16). Patients with disparate clinical histories and substantially different morbidities may therefore experience the development of valvular calcification. The characterization of specific biomarkers to follow the progression of the Calcific Aortic Valve disease is greatly needed. In our study, we show direct correlation of NT-pro-BNP, BNP and Osteopontin and the presence of CAS while Fetuin A levels an inverse relation. These results are in concordance with the published literature (9,17,18,19 and 20). It is important to note that this is the first study in which the most significant biomarkers used in the staging of CAS have been collectively compared in the same cohort of patients. This is a first step towards the development of a battery of biomarkers that could become useful in staging patients with aortic stenosis.

We are currently developing a biomarker discovery program based on a proteomic platform. The generation of a serum profile to indentifying stages of progression of Calcific aortic stenosis is, at the present time, far from ready to give any answers that could have clinical implications and will go through several trial and error periods during which different biomarker candidates will be abandoned and others incorporated. Eventually, the pathogenesis will be better elucidated allowing us to measure in the blood the different stages involved in this process. Our ability of doing so, will enhance the current evaluation of patients suspected of developing CAS and could be used in conjunction with clinical, genetic (21), epidemiologic and echocardiographic criteria.

Likely there are several phases that overlap with each other over time in the development of degenerative aortic valve disease. Identifying when inflammation is starting and when calcium deposits start to accumulate using serum biological markers in addition to imaging techniques would be an important first step in trying to better understand this process with the aim of a future therapeutic intervention. The more we learn about the natural course of aortic valve degeneration the better chance we have to successfully intervene before irreversible valve deterioration has ensued and surgery become necessary. Knowledge from this work could also be translated to patients who have already undergone aortic valve

replacement. In those patients, the systemic process that caused the initial valvular degeneration remains untreated leaving the prosthetic valve vulnerable to early degeneration.

## Acknowledgments

This project is currently supported by Award Number RC1HL100035 from the National Heart, Lung, And Blood Institute, NIH and by the “Harrison Memorial Fund” of the University Of Pennsylvania School Of Medicine.

## References

1. Freeman RV, Otto CM. Spectrum of calcific aortic valve disease: pathogenesis, disease progression, and treatment strategies. Circulation 2005;111:3316–26. [PubMed: 15967862]
2. Goldbarg SH, Elmariah S, Miller MA, Fuster V. Insights into degenerative aortic valve disease. J Am Coll Cardiol 2007;50:1205–13. [PubMed: 17888836]
3. Bach DS, Radeva JI, Birnbaum HG, Fournier AA, Tuttle EG. Prevalence, referral patterns, testing, and surgery in aortic valve disease: leaving women and elderly patients behind? J Heart Valve Dis 2007;16:362–9. [PubMed: 17702360]
4. Cowell SJ, Newby DE, Boon NA, Elder AT. Calcific aortic stenosis: same old story? Age Ageing 2004;33:538–44. [PubMed: 15308457]
5. Balmer C, Beghetti M, Fasnacht M, Friedli B, Arbenz U. Balloon aortic valvoplasty in paediatric patients: progressive aortic regurgitation is common. Heart 2004;90:77–81. [PubMed: 14676250]
6. O’Brien KD. Pathogenesis of Calcific Aortic Valve Disease: a disease process comes of age (and a good deal more). Arterioscler Thromb Vasc Biol 2006;26:1721–8. [PubMed: 16709942]
7. Gottdiener JS, Bednarz J, Devereux R, et al. American Society of Echocardiography recommendations for use of echocardiography in clinical trials. J Am Soc Echocardiogr 2004;17:1086–119. [PubMed: 15452478]
8. Rosenhek R, Binder T, Porenta G, et al. Predictors of outcome in severe, asymptomatic aortic stenosis. N Engl J Med 2000;343:611–7. [PubMed: 10965007]
9. Price PA, Lim JE. The inhibition of calcium phosphate precipitation by fetuin is accompanied by the formation of a fetuin-mineral complex. J Biol Chem 2003;278:22144–52. [PubMed: 12676929]
10. Straumann E, Meyer B, Misteli M, Blumberg A, Jenzer HR. Aortic and mitral valve disease in patients with end stage renal failure on long-term haemodialysis. Br Heart J 1992;67:236–9. [PubMed: 1554541]
11. Novaro GM, Aronow HD, Mayer-Sabik E, et al. Plasma homocysteine and calcific aortic valve disease. Heart 2004;90:802–3. [PubMed: 15201256]
12. Gunduz H, Arine H, tamer A, et al. The relationship between homomcysteine and calcific aortic valve stenosis. Cardiology 2005;103:207–11. [PubMed: 15838165]
13. Cho HJ, Cho HJ, Kim HS. Osteopontin: a multifunctional protein at the crossroads of inflammation, atherosclerosis, and vascular calcification. Curr Atheroscler Rep 2009;11:206–13. [PubMed: 19361352]
14. Jeevanantham V, Singh N, Izuora K, D’Souza JP, Hsi DH. Correlation of high sensitivity C-reactive protein and calcific aortic valve disease. Mayo Clin Proc 2007;82:171–4. [PubMed: 17290723]
15. Ross J Jr, Braunwald E. Aortic Stenosis. Circulation 1968;38:61–7. [PubMed: 4894151]
16. Atalar E, Ozturk E, Ozer N, Haznedaroglu IC, Kepez A, Coskun S, Aksoyek S, Ovunc K, Kes S, Kirazli S, Ozmen F. Plasma soluble osteopontin concentrations are increased in patients with rheumatic mitral stenosis and associated with the severity of mitral valve calcium. Am J Cardiol 2006;98:817–20. [PubMed: 16950193]
17. Yu PJ, Skolnick A, Ferrari G, et al. Correlation between plasma osteopontin levels and aortic valve calcification: potential insights into the pathogenesis of aortic valve calcification and stenosis. J Thorac Cardiovasc Surg 2009;138:196–9. [PubMed: 19577079]
18. Bergler-Klein J. Natriuretic peptides in the management of aortic stenosis. Curr Cardiol Rep 2009;11:85–93. [PubMed: 19236823]

19. Spargias K, Alexopoulos E, Thomopoulou S, et al. Effect of balloon valvuloplasty in patients with severe aortic stenosis on levels of N-terminal pro-B-type natriuretic peptide. Am J Cardiol 2009;104:846–9. [PubMed: 19733722]

20. Orlowska-Baranowska E, Baranowski R, Greszata L, et al. Brain natriuretic peptide as a marker of left ventricular hypertrophy in patients with aortic stenosis. J Heart Valve Dis 2008;17:598–605. [PubMed: 19137789]

21. Ix JH, Chertow GM, Shlipak MG, et al. Association of fetuin-A with mitral annular calcification and aortic stenosis among persons with coronary heart disease: data from the Heart and Soul Study. Circulation 2007;115:2533–9. [PubMed: 17485576]

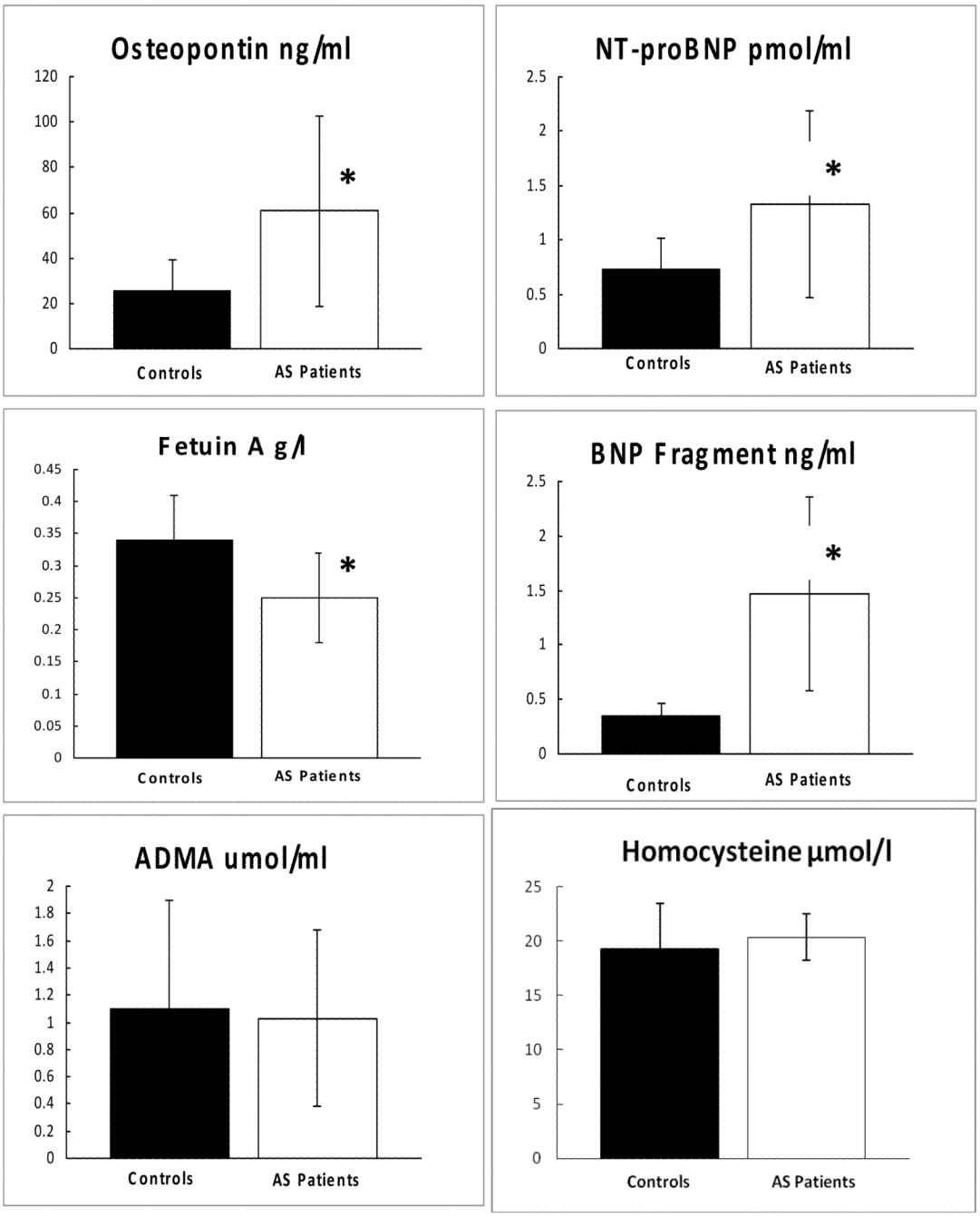


**Figure I.**

Plasma biomarker levels in CAS patients and controls

Osteopontin, pro-BNP, BNP fragment and Fetuin A levels were significantly elevated ($p<0.05$) in the CAS patients compared to the controls. Error bars represent ± SD.

**Table I**

Patients Baseline Characteristics

| Characteristics | Aortic Stenosis (N=33) | Controls (N=11) | Significance (p-value) |
|---|---|---|---|
| Age (years) | 75.9±7.17 | 55.36±24.15 | <0.001 |
| Male | 15 (45.5%) | 8 (72.72%) | 0.12 |
| Smokers | 11 (33.3%) | 1 (9.09%) | 0.12 |
| Hypertension | 27 (81.8%) | 7 (63.63%) | 0.21 |
| Diabetes Mellitus | 12 (36.36%) | 3 (27.27%) | 0.58 |
| CVA | 1 (3.03%) | 1 (9.09%) | 0.40 |
| PVD | 1 (3.03%) | 1 (9.09%) | 0.40 |
| Coronary Artery Disease | 8 (24.24%) | 1 (9.09%) | 0.28 |
| Hyperlipidemia | 20(62.6%) | 5 (45.45%) | 0.38 |
| AVC Score | 3.290.74 | 1.3 ± 0.5 | <0.001 |
| Adjusted Calcium mg/dL) | 9.2 ± 0.5 | 9.3 ± 0.6 | 0.58 |
| CaxP | 32.7±5.0 | 31.3±6.7 | 0.46 |

CVA: Cerebral vascular accident, PVD: peripheral vascular disease, CaxP: Calcium phosphorus product

## Table II

Biomarkers for Calcific Aortic Stenosis

| Biomarker | Presence/Severity | Prognosis |
|---|---|---|
| ADMA | + | n/a |
| CaxP | + | n/a |
| CRP | +/?* | + |
| Fetuin-A | + | n/a |
| GGT | +/−° | n/a |
| Homocysteine | ? | n/a |
| LDL | ? | − |
| Leptin | + | n/a |
| Natriuretic peptides | + | + |
| Osteopontin | + | n/a |
| tPA | + | n/a |

"+" indicates positive correlation; "−" indicates no correlation; "?" indicates contrary published results and "n/a" indicates that no studies were published on this issue.

° GGT correlates with AVS but not AVSc

* CRP correlates with AVS but unclear results for AVSc.

**Table III**

Mean levels of plasma biomarkers in patients with CAS and controls

| Subjects | Osteopontin ng/ml | Fetuin A g/l | NT-proBNP pmol/ml | BNP Fragment ng/ml | ADMA µmol/l | Homocysteine µmol/l |
|---|---|---|---|---|---|---|
| Controls | 25.42 ±13.79 | 0.34 ± 0.12 | 0.73 ± 0.29 | 0.35 ± 0.12 | 1.10± 0.08 | 19.23±4.19 |
| AS Patients | 60.79 ±42.05 | 0.25 ± 0.07 | 1.33 ± 0.86 | 1.47 ± 0.89 | 1.08 ± 0.65 | 20.34±2.14 |