# Natural Anti-Endothelial Cell Antibodies in Patients Undergoing Coronary Angiography

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**ABSTRAC.** **Background:** Anti-endothelial cell antibodies (AECA) are a known biomarker of endothelial dysfunction and damage in clinical practice, especially in autoimmune disease.

**Objectives:** To determine the relation between natural AECA levels and prognosis related to coronary artery disease.

**Methods:** Candidates for coronary angiography were prospectively enrolled. AECA levels were determined by ELISA assay. Mortality was evaluated after more than 5 years follow-up.

**Results:** Of a total 857 patients, 445 had high AECA levels (group 1) and 412 had low levels (< 1 OD unit, group 2). Both groups did not differ in age, sex, or presence of diabetes. The median follow up was 2293 days (76 months). Patients with high AECA levels were more likely to have normal coronary arteries on angiography (21.6% vs. 16.9%, *P* = 0.047) and less likely to have calcified lesions (19.0% vs. 26.6%, *P* = 0.028) and lower prevalence of abnormal renal functions (71.1 mg/dl vs. 66.5 mg/dl, *P* = 0.033). Patients with higher AECA levels had lower mortality levels (20.1% vs. 27.6%, *P* = 0.006). A logistic regression model demonstrated independent association between lower AECA levels and the presence of coronary atherosclerosis based on angiogram.

**Conclusions:** After a median of more than 6 years, higher natural AECA levels were associated with less coronary artery disease and lower mortality rates in patients undergoing coronary angiography.

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**KEY WORDS.** anti-endothelial cell antibodies (AECA), atherosclerosis, biomarkers, coronary artery disease

Multiple biomarkers in clinical practice are used as a complementary strategy to physical signs and imaging [1,2]. These assessments help in acute or chronic disease phase management, outcome prediction, or disease activity. Biomarkers are also important in screening the overall healthy general population to identify cohorts at risk and initiate preventive programs that are more effective and economically feasible [3-5]. An ideal biomarker should be able to objectively quantify, measure, and evaluate normal biological processes, pathogenic processes, or pharmacologic responses to a therapeutic intervention. In current clinical practice, the biomarker armamentarium is not robust enough to be used successfully for the abovementioned goals.

Anti-endothelial cell antibodies (AECA) are natural antibodies, and have been studied for more than 20 years [6-8]. Natural antibodies are antibodies that are present in the serum of healthy individuals in the absence of deliberate immunization with the target antigen [7-10]. The potential pathogenic role of AECAs in diseases involving the vascular system remains controversial [9,10]. Natural AECAs present in healthy individuals may be protective [11-13]. Such AECAs may participate in the host's defense against pathogens by opsonization, contribute to the clearance of senescent cells and immune complexes, and/or exert anti-inflammatory properties [14]. Circulating autoantibodies fulfilling the AECA definition are present in therapeutic preparations of normal human IgG (IVIg) and are internalized and induce an anti-inflammatory endothelial cell (EC) functional phenotype both on resting and activated ECs. In preliminary small studies, AECA were not associated with an accelerated atherosclerotic coronary artery disease [15,16].

Coronary artery disease (CAD) is the most significant cause of morbidity and mortality worldwide in the adult population with atherosclerosis [17]. It is by far the most preventable subjacent process [18-20]. Atherosclerosis is a chronic inflammatory response to different injury triggers. There are multiple experimental biomarkers involved in pathophysiology of atherosclerosis or are surrogate markers of the inflammatory activity, such as C-RP, IL-6, or IL-1ß.

In a large population with prolonged follow-up appointments, we studied the correlation between AECA levels and the presence and extent of coronary artery diseases and their clinical outcomes.

## PATIENTS AND METHODS

### STUDY DESIGN

We conducted a single center prospective observational study. We consecutively enrolled patients who were to undergo coronary angiography in our institution. The institutional review board approved the study. All patients gave their informed consent prior to their inclusion in the study. Patient data were collected from the computerized patient medical records.

### PATIENT POPULATION

All consecutive patients undergoing coronary angiography in our institution during an 18-month period were recruited. Patients under 18 years of age, pregnant females, patients with a severe active underlying illness, patients presenting with cardiogenic shock, those requiring cardiopulmonary resuscitation, and patients that could not provide informed consent were excluded. Patients with non-detectable AECA levels were also excluded. The data included age, sex, coronary risk factors, and detailed history of coronary artery disease, presence of atrial fibrillation included CHA2DS2-VASc score calculations, heart failure, previous stroke, and peripheral arterial disease. Laboratory and echocardiographic findings, coronary angiography indications, and results of the intervention were collected. Obstructive CAD was defined as luminal stenosis of ≥ 50% in the left main coronary artery (LMCA) and ≥ 70% in the main vessels and first degree side branches. Calcified coronary tree was visually identified by the interventional cardiologist performing the angiography. Patient survival was determined with the help of the national population registry, which was available for all patients.

### AECA DETECTION

For detection of AECA was detected using acyto-ELISA as previously described by Damianovich and colleagues [21]. Briefly, human umbilical vein endothelial cells (HUVEC) were seeded in gelatin-coated 96-well microtiter plates (Nunclon, Uppsala, Sweden) at 2.5 XIO4 cells/well and allowed to grow to confluence for 1 or 2 days. Cells were washed with Hanks' Balanced Salt Solution (HBSS) and incubated with blocking buffer (HBSS/O.5% BSA) for 30 min at 37°C to prevent nonspecific binding of Ab. After additional washing, HUVEC were exposed to normal human IgG and mouse sera (diluted 1/25 in HBSS/ lO% FCS) for 60 minutes at room temperature. Cells were washed again and incubated with the second Ab, alkaline phosphatase-conjugated goat anti-human or anti-mouse IgG (Jackson ImmunoResearch Laboratory, West Grove, PA, USA) followed by p-nitrophenyl phosphate disodium (Sigma, Rehovot, Israel) as a substrate. The OD was read at 405 nm in an ELISA plate reader (EAR 400 AT, SLT-Lab instruments, Austria). AECA levels were analyzed in the whole patient cohort, which was later divided into two groups: low titers and high titers according to AECA values.

### STATISTICAL ANALYSIS:

Baseline patient clinical characteristics and procedural data were compared between the patients in the two groups. The chi-square test and Fisher's exact test were used for dichotomous variables, and an independent *t*-test was used for continuous variables. Data are expressed as mean ± SD or frequency and percentage when appropriate.

The multivariate analysis was performed using the logistic regression model. A $P$ value < 0.05 was considered significant. Statistical analyses were performed using IBM Statistical Package for the Social Sciences statistics software, version 21 (SPSS, IBM Corp, Armonk, NY, USA).

## RESULTS

### BASELINE VARIABLES

During the study period, 857 consecutive patients were enrolled. The distribution of AECA levels range from minimum of 0.00005 to a maximum of 2.0 (mean 1.047 and median 0.857, standard deviation 0.853, variance 0.727, standard error 0.291) OD units. After assessing these levels, based on the median value of 0.857, for the sake of simplicity a level of 1.0 OD was used as the discriminative cut-off point to distinguish high versus low values, according the c-statics analysis, obtained in the ROC area.

Patient baseline characteristics are summarized in Table 1. The patient cohort was divided according the discriminative cut-off point calculated. Group 1 included patients with AECA level >1 (OD) and in group 2 the values < 1 (OD). The groups did not differ in age, sex, or other risk factors. Patients from group 1 were less likely to have preserved left ventricular systolic function. Patients from group 1 had higher total cholesterol, but did not differ in LDL cholesterol levels. The prevalence of abnormal renal functions as determined by an estimated adjusted GFR < 60 ml/min, was significantly lower in group 1 patients. Group 1 patients with higher levels of natural AECA were more likely to have normal coronary arteries on angiography and less likely to have calcified lesions and aortic valve stenosis. Patients in group 1 had lower mortality (20.1% vs. 27.6%, $P = 0.006$) [Table 3]. C statistics for AECA < 1 OD for mortality demonstrated an area under the curve of 0.543 [Figure 1].

### UNIVARIATE AND MULTIVARIATE ANALYSIS

We performed a univariate analysis of the effect of the demographic, clinical, laboratory, and angiographic variables on mortality [Table 2]. The deceased patients were older, had more co-morbidities, decreased renal function, lower left ventricular ejection fraction (LVEF) and hemoglobin, and higher prevalence of coronary atherosclerosis and especially coronary calcifications. Both lower absolute AECA levels and levels below 1 OD were predictive of mortality. Presence of obstructive CAD and acute coronary syndrome (ACS) as the reason for coronary angiography did not influence mortality [Table 2].

We conducted a multivariate analysis [Table 3] model, which combined age, CHA2DS2-VASc scores, presence of ACS, LVEF, elevated AECA levels (> 1 OD unit), and the presence of renal insufficiency (EGFR < 60 ml/min). We found that an elevated AECA level was independently associated with the presence of normal coronary angiogram (odds ratio 1.74, 95% confidence interval 1.12–2.70, $P < 0.014$).However, the levels of AECA were not independently associated with mortality [Table 3].

**Table 1.** Baseline patient's characteristics according to the AECA level group 1= AECA > 1 vs. group 2 AECA < 1 OD units

| Variable | All Patients (n=857) | AECA > 1 (n=412) | AECA < 1 (n=445) | *P* value |
|---|---|---|---|---|
| Age, years | 68.3 ± 11.6 | 68.2 ± 11.7 | 68.3 ± 11.6 | 0.886 |
| Sex, female | 30.1 | 30.1 | 30.1 | 0.996 |
| Diabetes mellitus | 44.5 | 40.3 | 48.2 | 0.055 |
| Hypertension | 77.0 | 78.4 | 75.6 | 0.336 |
| Old infarction | 22.1 | 19.7 | 24.1 | 0.126 |
| Previous CVA | 10.1 | 10.4 | 9.8 | 0.769 |
| PVD | 6.1 | 6.4 | 5.9 | 0.799 |
| Heart failure | 13.4 | 14.3 | 12.6 | 0.473 |
| Chronic kidney disease | 19.8 | 18.8 | 20.6 | 0.504 |
| Acute coronary syndrome | 49.0 | 51.7 | 46.5 | 0.14 |
| Abnormal coronary angiography | 80.9 | 78.4 | 83.1 | 0.047 |
| Obstructive CAD | 57.3 | 55.1 | 59.3 | 0.119 |
| Calcified coronary tree | 23.1 | 19.0 | 26.6 | 0.028 |
| Elevated LV filling pressure | 32.2 | 32.7 | 31.8 | 0.820 |
| EF>50 | 49.3 | 43.3 | 54.3 | 0.005 |
| Preserved LV function | 49.3 | 43.3 | 54.3 | 0.005 |
| Aortic stenosis | 19.1 | 16.0 | 21.7 | 0.041 |
| Mortality | 24.0 | 20.1 | 27.6 | 0.006 |
| Atrial fibrillation | 19.5 | 19.8 | 19.2 | 0.820 |
| CHA2DS2-VASc | 3.4 ± 1.7 | 3.3 ± 1.7 | 3.4 ± 1.7 | 0.467 |
| Creatinine | 1.18 ± 1.16 | 1.19 ± 1.02 | 1.18 ± 1.29 | 0.887 |
| Adjusted GFR | 68.7 ± 31.1 | 71.1 ± 32.9 | 66.5 ± 29.1 | 0.033 |
| Hemoglobin | 13.2 ± 1.6 | 13.2 ± 1.6 | 13.1 ± 1.6 | 0.49 |
| Troponin I | 1.75 ± 5.09 | 2.31 ± 5.6 | 1.42 ± 4.74 | 0.065 |
| Total cholesterol | 167.5 ± 45.3 | 172.4 ± 45.9 | 163.0 ± 44.4 | 0.015 |
| LDL cholesterol | 97.3 ± 33.4 | 97.7 ± 33.7 | 97.0 ± 34.2 | 0.852 |
| Ejection fraction | 49.4 ± 9.8 | 48.1 ± 10.5 | 50.5 ± 9.1 | 0.002 |
| Hemoglobin A1c | 7.2 ± 2.0 | 6.8 ± 1.2 | 8.8 ± 3.7 | 0.252 |

AECA = anti-endothelial cell antibodies, CAD = coronary artery disease, CVA = cerebrovascular accident, LDL= low-density lipoproteins, LV = left ventricle, PVD = peripheral vascular disease

Dichotomous variables are given as percentage of total in each group. Continuous values are presented as mean ± standard deviation.

**Table 2.** Univariate analysis of effect on mortality

| Variable | Total patients (%) | Alive | Deceased | *P* value |
|---|---|---|---|---|
| AECA > 1 | 48.1 | 50.5 | 40.4 | 0.0006 |
| Age, years | 68.3 ± 11.6 | 66.1 ± 11.4 | 75.1 ± 9.6 | < 0.0001 |
| Sex, female | 30.1 | 29.2 | 33.0 | 0.297 |
| Diabetes mellitus | 44.5 | 41.8 | 52.9 | 0.008 |
| Hypertension | 77.0 | 74.4 | 84.9 | 0.002 |
| Old infarction | 22.1 | 22.4 | 20.9 | 0.647 |
| Previous CVA | 10.1 | 9.1 | 13.4 | 0.072 |
| PVD | 6.1 | 3.8 | 13.4 | < 0.0001 |
| CHF | 13.4 | 9.9 | 24.4 | < 0.0001 |
| Chronic kidney disease | 19.8 | 13.7 | 38.9 | < 0.0001 |
| Acute coronary syndrome | 49.0 | 47.5 | 53.6 | 0.139 |
| Atrial fibrillation | 19.5 | 15.5 | 32.0 | < 0.0001 |
| Abnormal coronary angiography | 80.9 | 78.7 | 87.4 | 0.005 |
| Obstructive CAD | 57.3 | 56.3 | 60.3 | 0.308 |
| Calcified coronary tree | 23.1 | 19.6 | 33.2 | < 0.0001 |
| Elevated LV filling pressure | 32.2 | 23.2 | 54.2 | < 0.0001 |
| EF>50 | 49.3 | 59.9 | 47.3 | 0.004 |
| Aortic stenosis | 19.1 | 13.6 | 33.9 | < 0.0001 |
| CHA2DS2-VASc | 3.4 ± 1.7 | 3.1 ± 1.7 | 4.2 ± 1.6 | < 0.0001 |
| Creatinine | 1.18 ± 1.16 | 1.06 ± 1.02 | 1.57 ± 1.45 | < 0.0001 |
| Adjusted GFR | 68.7 ± 31.1 | 74.6 ± 29.5 | 50.3 ± 28.7 | < 0.0001 |
| Hemoglobin | 13.2 ± 1.6 | 13.4 ± 1.5 | 12.4 ± 1.6 | < 0.0001 |
| Troponin I | 1.75 ± 5.09 | 1.62 ± 5.10 | 2.08 ± 5.07 | 0.368 |
| Total Cholesterol | 167.5 ± 45.3 | 168.5 43.6 | 164.6 ± 49.5 | 0.393 |
| LDL Cholesterol | 97.3 ± 33.4 | 98.0 ± 33.7 | 95.0 ± 34.7 | 0.508 |
| Ejection fraction | 49.4 ± 9.8 | 50.4 ± 9.2 | 46.8 ± 11.0 | < 0.0001 |
| Hemoglobin A1c | 7.2 ± 2.0 | 6.9 ± 1.6 | 8.5 ± 2.9 | 0.227 |
| AECA | 1.05 ± 0.85 | 1.09 ± 0.86 | 0.94 ± 0.83 | 0.023 |

AECA = anti-endothelial cell antibodies, CAD = coronary artery disease, CVA = cerebrovascular accident, LDL= low-density lipoproteins, LV = left ventricle, PVD = peripheral vascular disease

Dichotomous variables are given as percentage of total in each group. Continuous values are presented as mean ± standard deviation.

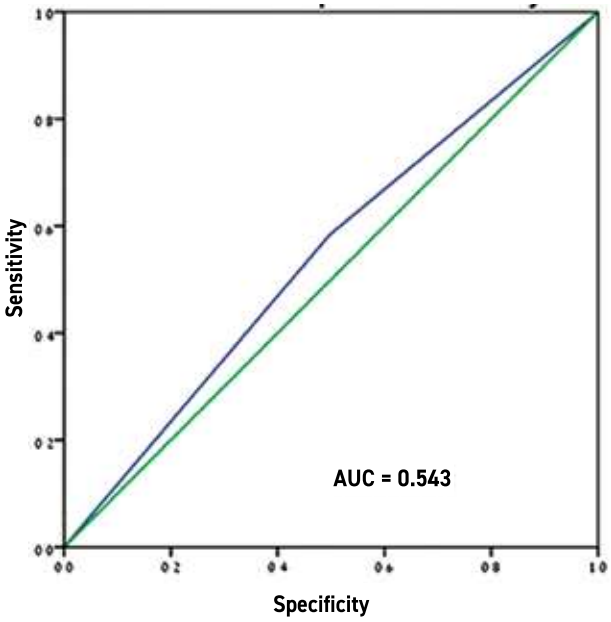
**Table 3.** Independent predictors of abnormal coronary angiograms

| Variable | Hazard ratio | 95% confidence interval | *P* value |
|---|---|---|---|
| **Logistic regression** | | | |
| AECA < 1.0 | 1.74 | 1.12–2.70 | 0.014 |
| LVEF (per 1% decrease) | 1.03 | 1.01–1.05 | 0.037 |
| Acute coronary syndrome present | 2.81 | 1.78–4.42 | < 0.0001 |
| CHA2DS2-VASc score | 1.55 | 1.32–1.83 | < 0.0001 |
| GFR < 60 ml/min | 2.35 | 1.39–3.97 | 0.001 |
| Age (per 1 year increase) | 1.01 | 0.98–1.04 | 0.496 |
| **Cox regression of independent predictors of mortality** | | | |
| AECA < 1.0 | 1.02 | 0.75–1.34 | 0.889 |
| LVEF (per 1% decrease) | 1.03 | 1.01–1.04 | < 0.0001 |
| Acute coronary syndrome present | 1.04 | 0.76–1.41 | 0.804 |
| CHA2DS2-VASc score (per 1 increase) | 1.23 | 1.10–1.37 | < 0.0001 |
| GFR < 60 ml/min | 2.20 | 1.46–3.31 | < 0.0001 |
| Age (per 1 year increase) | 1.03 | 1.01–1.05 | 0.007 |

AECA = anti-endothelial cell antibodies, CAD = coronary artery disease, CVA = cerebrovascular accident, GFR = glomerular filtration rate, LDL= low-density lipoproteins, LV = left ventricle, LVEF = left ventricular ejection fraction, PVD = peripheral vascular disease

**Figure 1.** ROC curve AECA < 1.0 as predictor of mortality

AECA = anti-endothelial cell antibodies, AUC = area under the curve, C statistics




Diagonal segments are produced by ties

The mean and median follow-up was 1745 days and 2293 days, respectively.

## DISCUSSION

We found an association between low levels of AECA and the presence of CAD. This finding is important because AECA levels are easily obtained and can be used as a biomarker for clinical prognosis and risk stratification.

Biomarkers are some of the most objective, quantifiable medical signs that we can reproducibly measure. The use of biomarkers in clinical research is somewhat newer, and the best approaches to this practice are still being developed and refined [22].

AECAs are a heterogeneous group of natural antibodies that react to a diverse array of endothelial cell (EC) bound antigens [10,11], and the biological effect achieved is related to the targeted antigen [23]. Natural AECA levels represent the vascular wall protection or damage activity according the antigen studied, similar to the measurement of C-RP inflammatory activity.

These finding are not aligned with previous studies. Farsi A. et al. [15] showed the opposite finding with regard to the association between high AECA levels with CAD in a small group of unstable angina patients. However, we previously found a lack of correlation between AECA levels and coronary atherosclerosis visualized in coronary angiography [16]. These discrepancies can be explained in part by the different ELISA assay used in each study and the different clinical presentations. In our current study, the subjects were consecutively enrolled for cardiac catheterization and the cohort was significantly larger than in previous studies as was the prospective follow-up for outcome measures.

In this study, we proposed to re-float AECA, an old well-studied antibody in the last 20 years in the biomarkers armamentarium profile. We think that a representative biomarkers panel can help in the preventive and risk stratification in the general population, assumed as healthy subjects. Recently, Ridker and co-authors [24] tested successfully in CANTOS trial the therapeutic intervention with canakinumab in patients with increased levels of high sensitive C-reactive protein. We propose that specific AECA may have potential value in therapeutics paths. The measured response may be functional and physiological, biochemical at the cellular level, or a molecular interaction. We used a cut-off AECA level of > 1 and < 2 to describe the natural AECA levels. After a median follow up for more than 6 years we found that this cut-off discriminates between patients with angiographically normal coronary tree and those with pathological

coronary arteries. This finding is enhanced by other significant observations correlated with atherosclerosis process or complications, as more frequent coronary artery calcifications, aortic stenosis and impaired renal function. Moreover, we found that natural antibodies correlated with lower mortality. These observations raise the hypothesis that AECA could play a protective role. We can speculate that this functional property could result from interference with ligands that act to activate endothelial cell via engagement of membrane proteins. As the spectrum of AECA is very large, subset of anti-endothelial antibodies could target different ligands and either directly activation endothelial cells [21] or protected against ligand mediated activation.

## STRENGTHS AND LIMITATIONS

Our study is notable for the prolonged follow-up duration. The major study limitation is the lack of full characterization of AECA with regard to their binding properties. We did not analyze the interaction of between AECA levels with other laboratory biomarkers.

## CONCLUSIONS

High levels of natural AECA in reduced angiographically evident coronary atherosclerosis and lower mortality in more than 6-year median follow-up. These findings will be the basis for exploring various subgroups of AECA that may have atheroprotective properties.

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**Much of writing might be described as mental pregnancy with successive difficult deliveries.**

John Boynton Priestley (1894–1984), English novelist, playwright, screenwriter, broadcaster and social commentator

**A man's life is interesting primarily when he has failed -- I well know. For it is a sign that he has tried to surpass himself.**

Georges Eugène Benjamin Clemenceau (1841–1929),
French statesman who served as prime minister of France from 1906 to 1909 and again from 1917 until 1920