Material and methods

Study Population. A retrospective study was performed on a total of 338 patients enrolled at the University of Pennsylvania according to the approved IRB protocol #809349 (**Figure 1**). All patients included in this study have been followed for aortic valve diseases (stenosis or insufficiency) and/or enlargement of the ascending aorta and reached the criteria for surgical intervention. Blood was taken before surgery and all patients provided written informed consent. Surgical patients were divided in two groups, according to the morphology of the aortic valve assessed by transesophageal echocardiography (TTE), computed tomography (CT scan) or both, and confirmed by intra-operatory observation. Exclusion criteria were: genetic disease associated with TAA, connective tissue disease, chronic inflammatory disease, previous myocardial infarction, severe heart failure (NYHAIII+, IV), endocarditis and active cancer. A total of 135 patients met the inclusion criteria, n=74 with BAV and n=61 with TAV. Among them 32 BAV and 33 TAV underwent aortic valve replacement without aortoplasty (BAVAVR). 42 BAV and 29 TAV underwent aortic valve repair or replacement combined with an ascending aorta surgery (repair or replacement) (BAVAVR/AA or TAVAVR/AA). A detailed description the patients' demographics is summarized in Table 1 and 2.

Aortic tissue collection. Ascending aorta was excised from BAV patients undergoing aortic valve replacement combined with ascending aorta surgery. A small fragment of ascending aorta was also obtained from patients with TAV undergoing aortic valve replacement only.

Immunohistochemistry and Immunofluorescence of OCT sections. Fresh ascending aorta tissues were collected during surgery, fixed in formalin and embedded in OCT. Hematoxylin & Eosin and Modified Movat's Pentachrome staining were performed on 6μm sections by the MCRC histology core at the University of Pennsylvania. Black stain indicates nuclei and elastic fibers; yellow stain indicates collagen fibers; blue stain indicates proteoglycans and glycosaminoglycans; red indicates muscular tissue. Immunohistochemistry and/or immunofluorescence for s100A12, HMGB-1, RAGE and CD45 were performed following standardized protocols.

sRAGE quantification. Blood was collected prior to surgery from the patients and processed to obtain serum and plasma then stored at -80°C until the assay was performed. Plasma analysis for sRAGE, s100A12 and HMGB-1 level was conducted using ELISA kits (R&D Systems and MBL International).

Western Blotting analysis of human ascending aorta. Whole tissue extract was obtained from frozen ascending aorta tissues. Protein expression was determined using antibodies against RAGE (ab3611), HMGB-1(ab18256) and GAPDH (ab9485) following standardized protocol[1].

Study Design. sRAGE levels were analyzed in two ways: First, comparisons were made between all BAV and TAV patients; and second, comparisons were made between BAV and TAV patients with ascending aorta diameter ≤ 4.5 cm. Linear regression was used to determine the relationship between sRAGE values and age, gender, diagnosis of CAD and diabetes and presence of common risks factor for cardiovascular disease (hypertension, hyperlipidemia, smoking). Comparisons were then made within the BAV group, (and within the TAV group) between patients undergoing AVR surgery only and patients undergoing AVR and ascending aorta surgery (AVR/AA). Linear regression was used to examine the relationship between sRAGE values and ascending aorta diameter and ratio between ascending aorta diameter/body surface area (BSA). BSA was calculated using the Mosteller formula. HMGB-1 and s100A12

plasma levels were also tested in a subgroup of patients with known sRAGE values (n=30 and n=38 patients respectively).

Statistical Analysis. The data were analyzed using SPSS software (version 19.1; IBM/SPSS, Chicago, IL) and SAS (version 9.2). Continuous variables are expressed as mean ± standard error (SEM). Comparisons of continuous variables between groups were performed with the Student's t test (in the case of patient age) or nonparametric (Mann-Whitney U test) tests to adjust for abnormalities in the distribution of other variables (sRAGE). Multivariate General Linear Model ANOVA was used to evaluate the relationship of factors and their interactions with the level of sRAGE. Univariate and multiple regression were used to evaluate the association of SRAGE as a predictive marker of BAV status. Differences were considered statistically significant at values of $p<0.05$. To determine the specificity and sensitivity of sRAGE quantification, area under the receiver operating characteristic curves (AUC of ROC curves) was calculated using statistical software GraphPad Prism 6. Regression analyses were performed using SAS (proc logistic) with goodness of fit testing according to the methods of Hosmer and Lemeshow.

Supplemental References

1. Branchetti E, Poggio P, Sainger R, Shang E, Grau JB, Jackson BM, Lai EK, Parmacek MS, Gorman RC, Gorman JH, Bavaria JE, Ferrari G. Oxidative stress modulates vascular smooth muscle cell phenotype via CTGF in thoracic aortic aneurysm. *Cardiovasc Res.* 2013 Nov 1;100:316-24