

## Hypertension Is Associated With Preamyloid Oligomers in Human Atrium: A Missing Link in Atrial Pathophysiology?

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**Background**—Increasing evidence indicates that proteotoxicity plays a pathophysiologic role in experimental and human cardiomyopathy. In organ-specific amyloidoses, soluble protein oligomers are the primary cytotoxic species in the process of protein aggregation. While isolated atrial amyloidosis can develop with aging, the presence of preamyloid oligomers (PAOs) in atrial tissue has not been previously investigated.

**Methods and Results**—Atrial samples were collected during elective cardiac surgery in patients without a history of atrial arrhythmias, congestive heart failure, cardiomyopathy, or amyloidosis. Immunohistochemistry was performed for PAOs using a conformation-specific antibody, as well as for candidate proteins identified previously in isolated atrial amyloidosis. Using a myocardium-specific marker, the fraction of myocardium colocalizing with PAOs (PAO burden) was quantified (green/red ratio). Atrial samples were obtained from 92 patients, with a mean age of  $61.7 \pm 13.8$  years. Most patients (62%) were male, 23% had diabetes, 72% had hypertension, and 42% had coronary artery disease. A majority ( $n=62$ ) underwent aortic valve replacement, with fewer undergoing coronary artery bypass grafting ( $n=34$ ) or mitral valve replacement/repair ( $n=24$ ). Immunostaining detected intracellular PAOs in a majority of atrial samples, with a heterogeneous distribution throughout the myocardium. Mean green/red ratio value for the samples was  $0.11 \pm 0.1$  (range 0.03 to 0.77), with a value  $\geq 0.05$  in 74 patients. Atrial natriuretic peptide colocalized with PAOs in myocardium, whereas transthyretin was located in the interstitium. Adjusting for multiple covariates, PAO burden was independently associated with the presence of hypertension.

**Conclusion**—PAOs are frequently detected in human atrium, where their presence is associated with clinical hypertension. (*J Am Heart Assoc.* 2014;3:e001384 doi: 10.1161/JAHA.114.001384)

**Key Words:** amyloid • atrial natriuretic peptide • atrium • preamyloid oligomers

The factors that generate the substrate for atrial arrhythmias are complex, and fundamental mechanisms of

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disease pathogenesis remain uncertain.<sup>1</sup> For example, the most important comorbidity contributing to atrial fibrillation (AF) is hypertension, accounting for >20% of cases.<sup>2</sup> Yet, in many hypertensive patients with AF, there is no evidence of atrial enlargement or fibrosis/pathology apparent by imaging.<sup>3,4</sup> Thus, the pathophysiologic mechanism or mechanisms in this circumstance are not known.

The accumulation of insoluble, misfolded proteins is linked to an increasing number of aging-related degenerative diseases, including Alzheimer disease and type 2 diabetes.<sup>5–7</sup> For amyloidoses, or the deposition of insoluble proteins that are fibrillar in nature,<sup>8</sup> a unifying feature is a common cross  $\beta$ -sheet structure that binds dyes such as Congo red. For systemic amyloidoses, the pathogenic mechanism is the large bulk of amyloid deposited into vital organs.<sup>9</sup> In contrast, for organ-specific amyloidoses such as Alzheimer, there is no detectable correlation between the quantity of fibrillar deposits and the stage of disease advancement.<sup>9,10</sup> Rather, disease phenotype correlates most closely with accumulation

of prefibrillar protein aggregates, rather than mature insoluble fibrils.<sup>5–7,10</sup> Preamyloid oligomers (PAOs) have been shown to be cytotoxic to multiple cell types,<sup>10</sup> including cardiomyocytes.<sup>11</sup> Importantly, PAOs derived from different proteins share a common structural epitope regardless of amino acid sequence, and possibly common pathogenic mechanisms. A critical distinction between soluble oligomers and amyloid deposits is that oligomers do not bind Congo red and hence are not visible by standard amyloid staining methods.

As in the brain, aging-related amyloidosis can be limited to the atrium, a condition known as isolated atrial amyloidosis.<sup>12,13</sup> The prevalence of this atrium-specific disorder increases with age, exceeding 90% in the ninth decade of life.<sup>12</sup> Previous studies indicated that amyloid deposits in isolated atrial amyloidosis are immunoreactive for atrial natriuretic peptide (ANP) in most patients, and occasionally for the plasma transport protein transthyretin (TTR).<sup>12,13</sup>

Recent studies provide mounting evidence for a role of proteotoxicity in heart disease, specifically ventricular dysfunction and cardiomyopathy.<sup>7</sup> Pioneering work has shown that mutations in the gene encoding  $\alpha$ B-crystallin, a small heat shock protein that binds desmin in striated muscle, cause a cardiomyopathy characterized by accumulation of PAOs and aggresomes, leading to congestive heart failure and premature death.<sup>14,15</sup> Moreover, PAOs have been shown to play a causative role in this cardiomyopathic process.<sup>16</sup> Similarly, investigators have found evidence of ventricular PAO formation in human dilated and hypertrophic cardiomyopathy.<sup>16</sup> Recent clinical findings have also linked proteotoxicity to idiopathic dilated cardiomyopathy,<sup>17</sup> while amyloidosis has now been documented in heart failure with preserved ejection fraction caused predominantly by hypertension.<sup>18</sup> Given that PAOs are the pathogenic species in organ-specific amyloidosis and that this process occurs in human atrium, we hypothesized that PAOs can develop in human atrial tissue in association with 1 or more conditions causing atrial pathology. To test this hypothesis, atrial samples were harvested from patients undergoing elective cardiac surgery and probed with an antibody recognizing the structural epitope common to PAOs. In this report, we investigate the clinical basis and molecular composition of atrial PAOs in this cohort of patients.

## Methods

### Tissue Acquisition

Human cardiac tissue from the right or left atrial free wall (RA or LA, respectively) was acquired at the time of elective cardiac surgery from patients enrolled in 2 randomized, double-blind, placebo-controlled clinical trials of postoperative AF: a National Institutes of Health–sponsored clinical trial (Renin-Angiotensin-Aldosterone System [RAAS], Inflammation,

and Postoperative Atrial Fibrillation, ClinicalTrials.gov #NCT00141778) in which patients were randomly assigned to ramipril, spironolactone, or placebo starting 1 week prior to elective cardiac surgery,<sup>19,20</sup> and the OPERA (Omega-3 Fatty Acids for the Prevention of Post-Operative Atrial fibrillation) trial<sup>21</sup> in which patients were randomized to perioperative treatment with fish oil or placebo. In both trials, the incidence of postoperative AF was the same on placebo as in the treatment arms. For this analysis, samples were included from patients with (1) no history of atrial arrhythmias, (2) well-preserved left ventricular function (left ventricular ejection fraction  $\geq 40\%$ ), (3) no symptoms of congestive heart failure, and (4) no history of systemic or cardiac amyloidosis. Informed consent was obtained using a protocol approved by the Vanderbilt Institutional Review Board. RA tissue was harvested during cannula placement for cardiopulmonary bypass, while LA tissue was removed from the edge of an LA incision required for surgery. Tissue was immediately fixed in 10% buffered formalin for 24 hours, dehydrated, and embedded in paraffin. Specimens were cut into 5- $\mu$ m-thick sections and mounted on glass slides.

### Immunohistochemistry

These methods have been described in detail previously.<sup>22</sup> In brief, immunostaining was performed using a mouse monoclonal antibody specific for striated muscle (MF-20, directed against the heavy chain of myosin II; 1:15, Developmental Studies Hybridoma Bank) to label myocardium, and a rabbit polyclonal antibody (A-11; 1:200, courtesy of Dr Charles Glabe) recognizing a conformational epitope common to all PAOs,<sup>6,23</sup> with secondary goat anti-mouse Alexa 568–conjugated and donkey anti-rabbit Alexa 488–conjugated antibodies (Molecular Probes), respectively. Negative control staining was also performed on separate sections with primary and secondary antibodies omitted, or nonspecific IgG substituted for primary antibody, with similar background results.

### Image Acquisition

Slides were imaged using a confocal microscope (LSM510) with a 20 $\times$ /0.75 Plan-Apochromat objective (Carl Zeiss, Inc). The confocal aperture was adjusted to restrict detection to optical sections of 4- $\mu$ m thickness (in the z-dimension), and a single image was acquired for each of 6 random regions per slide enriched for myocardium. Before data acquisition, the dynamic range for both the green (Alexa 488) and red (Alexa 568) channels was maximized to achieve the highest precision and contrast in the image. The background slide and its A-11/MF-20 counterpart were collected from serial sections for each sample and were processed and imaged in parallel using the same imaging configurations.

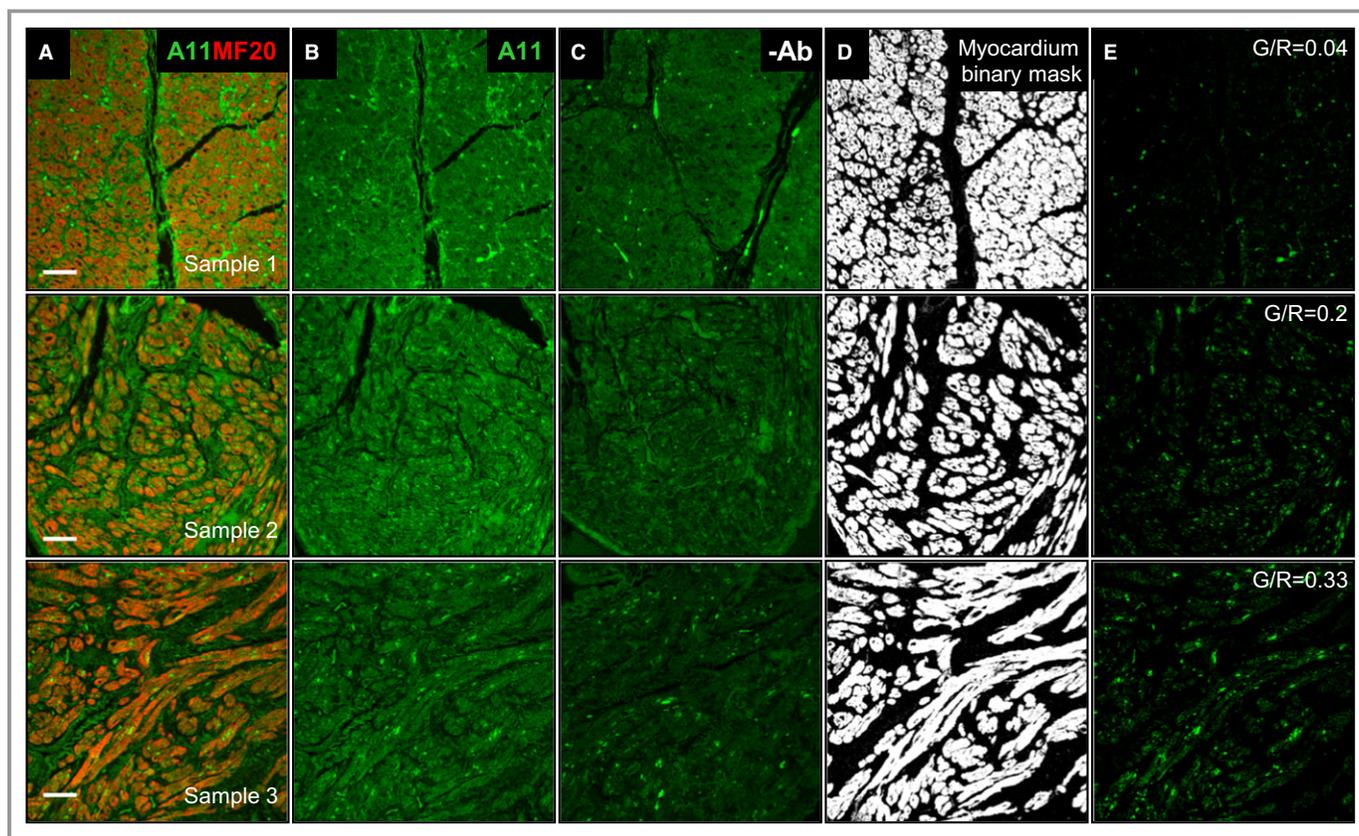
## Image Analysis and Quantification of PAO Burden

Because of heterogeneity in PAO myocardial distribution (Figure 1), we used a reproducible, previously validated method to quantify the relative myocardial surface area (red) that contained PAOs (green), or green/red ratio (G/R), as a *spatial representation* of PAO burden in an atrial sample.<sup>22</sup> In brief, antibody-negative control images were obtained concurrently with antibody-positive images using adjacent sections to enable threshold background subtraction and elimination of intensely autofluorescent, nonmyocardial signals (ie, red blood cells). All pixels with signal values between the range of the minimum and maximum threshold were defined as positive signal, independent of the absolute signal value. For the positive MF-20 image, a binary mask of the myocardial image was created using pixels with values in the thresholded range, and the total number of qualifying pixels was defined as the myocardial area (R). The positive MF-20 mask was overlaid with the background-subtracted positive A-11 image, and the area of myocardium (pixels) that also contained PAOs (positive

green signal) was measured (G). This provided the relative amount of myocardium containing positive A-11 signal, or G/R value. Using this semiautomated analytical method, quantitative analysis of this spatially heterogeneous structural abnormality can be performed in small atrial samples in a reproducible manner.<sup>22</sup>

## Immunohistochemistry for ANP and TTR

Adjacent sections of atrium were immunostained for A-11 and either ANP or TTR. For ANP immunostaining, the same protocol described here for A-11 was used with a rabbit polyclonal antibody directed against  $\alpha$ -ANP (1-28; 1:200, Phoenix Pharmaceuticals, Inc) along with MF-20. For TTR, a previously published protocol was used with modifications using a rabbit polyclonal anti-human-TTR (1:500; DakoCytomation).<sup>24</sup> For both proteins, a positive control preparation was generated by transfecting HEK or COS M6 cells with Myc-DDK-tagged human TTR (OriGene Technologies, NM\_000371) or human natriuretic peptide precursor A (NPPA NM\_006172.1), respectively. Western



**Figure 1.** Distribution of preamyloid oligomers (PAOs) in human atrium. Representative human atrial samples with a low (sample 1), medium (sample 2), and high (sample 3) green/red ratio (G/R) value are shown. Immunolabeling results with both myosin heavy chain-specific monoclonal antibody (MF-20) and PAO-specific antibodies (A-11; Column A), or A-11 alone (Column B). Column C. Background fluorescent signal after excitation using 488 nm in the presence of nonspecific IgG. Columns D, E. Images after creation of the myocardium binary mask and PAO signal within the myocardium (with G/R value), respectively. Scale bars=50  $\mu$ m.

blot-positive cells were centrifuged and embedded into paraffin.

## Alkaline Congo Red Staining

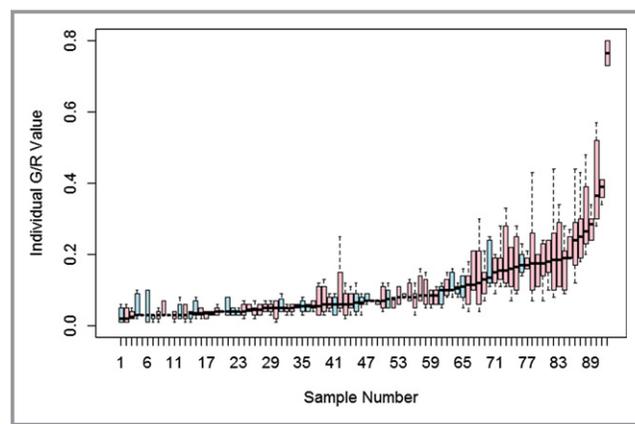
Tissue sections were stained in Congo red solution using standard methods. Positive controls with known amyloid were stained and examined concurrently, and they showed apple green birefringence under polarized light. Negative control samples were obtained from structurally normal hearts in patients with no known heart disease that were originally intended as donor hearts for cardiac transplantation but were rejected for technical reasons.

## Quantitation of Fibrosis

Atrial samples were sectioned (5  $\mu\text{m}$ ) and stained by using a standard Masson's trichrome procedure to visualize collagen-rich tissue. Digitized images of the entire specimen were acquired by using a Nikon AZ100M transmitted light microscope at a magnification of 2 $\times$  to assess the degree of interstitial fibrosis. Areas of normal collagen accumulation (ie, epicardium, endocardium, perivascular) were excluded. Analysis was performed by using Metamorph (Molecular Devices, Sunnyvale, CA), with color filters for red (myocardium) and blue (collagen) created for each image.<sup>25</sup> The percentage of myocardial fibrosis was calculated by dividing the number of blue pixels by the total number of blue and red pixels.

## Statistical Analysis

For G/R values, data from different patients were assumed to be statistically independent, whereas those within a patient were correlated. Data inspection revealed that the individual field G/R values of the 92 patients did not have homogeneous variance (Figure 2). Therefore, we used a linear mixed-effects model with random effect of study subject to handle the heterogeneity of variances within a patient. The correlation structure was based on the compound symmetry structure. The focus of the multivariable data analysis was to study the potential association between PAO burden, expressed as G/R values, and specific clinical variables, including age, sex, hypertension, body mass index,  $\beta$ -blocker therapy, atrial source, and cardiac substrate (specifically aortic valve [AV] replacement). The general linear model analysis was used to study the correlation between G/R values and percent fibrosis. The statistical tests for model parameters were 2-sided with a statistically significant level of .05. All statistical analysis was performed using R nlme package with version 2.14.1 for Windows.



**Figure 2.** Median and individual green/red ratio (G/R) values for all patient samples. For each patient sample ( $x$  axis), the range (vertical bars), median (black horizontal bars), and SD (dotted lines) of the individual imaging field G/R values ( $y$  axis) are illustrated. Whiskers are absent for data sets in which the 25% or 75% quantiles are equal or close to the median value. Data for patients with hypertension are shown in red, while blue indicates no history of hypertension.

## Results

### Patient Population

Atrial myocardium was harvested from 92 patients during cardiac surgery. Demographic features for the group are displayed in Table 1. Patients were predominantly male with a mean age of 61.7 years. Less than one-third of the patients had diabetes ( $n=21$ ), while a majority (72%) had hypertension, and nearly half (42%) had clinically significant coronary artery disease. By virtue of the exclusion criteria, left ventricular ejection fraction was well preserved in the cohort ( $58\pm 9\%$ ). Most patients underwent AV replacement (67%), with tissue harvested from the RA at the time of cannulation for cardiopulmonary bypass. Approximately one-third (36%) of patients developed postoperative atrial fibrillation (AF).

### Quantitative Analysis of PAO Burden

As illustrated in Figures 1 and 2, PAOs were identified in the atrial tissue of many patients. This abnormality was characterized by a nonhomogeneous or “patchy” distribution within the myocardial sample. For background adjusted images (Figure 1), PAO signal was detected within cytosolic regions of cardiomyocytes, as expected for soluble oligomers. For the entire patient population, the mean sample G/R value averaged  $0.11\pm 0.10$ , ranging from  $0.03\pm 0.1$  to  $0.77\pm 0.5$ , with a value of at least 0.05 (or 5% of myocardial area) for 74 of 92 patients. The within-sample heterogeneity is further displayed in Figure 2, which plots the individual field G/R values for all samples (range 0.03 to 0.80). The mean G/R

**Table 1.** Patient Characteristics (N=92)

Age, y	61.7±13.8*
Male sex, %	57 (62)
White, %	87 (95)
Hypertension, %	66 (72)
Diabetes, %	21 (23)
Coronary artery disease, %	39 (42)
BMI	29±5.7*
Left ventricular EF, %	58±9*
Medical therapy	
β-Blocker, %	39 (42)
ACE inhibitor, %	30 (33)
ARB, %	17 (18)
Surgical procedure (s)	
AV replacement, %	62 (67)
CABG, %	34 (37)
MV replacement	24 (26)
Other, %	5 (5)
Sample: right atrium, %	70 (76)
Postoperative AF, %	33 (36)

BMI indicates body mass index; EF, ejection fraction; ACE, angiotensin-converting enzyme; ARB, angiotensin II receptor blocker; AF, atrial fibrillation; AV, aortic valve; CABG, coronary artery bypass grafting; MV, mitral valve.

\*Mean±SD.

values for LA and RA samples were nearly identical ( $0.11±0.09$  and  $0.11±0.11$ , respectively). Based on the intrinsic composite error of the measurements,<sup>22</sup> G/R values  $<0.05$  (or 5% of myocardial area) are statistically indistinguishable from a value of 0; these low values were present in 18 patients, or 20% of the cohort.

To ensure that the A-11 antibody was detecting protein oligomers and not amyloid fibrils, Congo red staining was performed on 16 patient samples representing a range of mean G/R values ( $0.03±0.02$  to  $0.43±0.06$ ). Examination of all Congo red-stained sections under polarized light revealed no evidence of amyloid deposition (Figure 3B). These results further confirm that the protein oligomers identified in the atrial samples are not mature amyloid, given the negative Congo red staining.

Hematoxylin and eosin slides for representative samples having a range of G/R values (0.03 to 0.43;  $n=30$ ) were reviewed by a cardiac pathologist (J.B.A.). These revealed a spectrum of pathologic changes in human atrium typical of patients with the underlying cardiac diseases shown in Table 1, including focal areas of myocyte hypertrophy, myocytolysis, myocardial ischemia, and fibrosis, with no evidence of inflammation or abnormal storage disease. There was no

difference in the extent or spectrum of these changes observed in patients with low vs high G/R values, with representative images shown in Figure 3A.

### Comparison of PAO Burden and Fibrosis

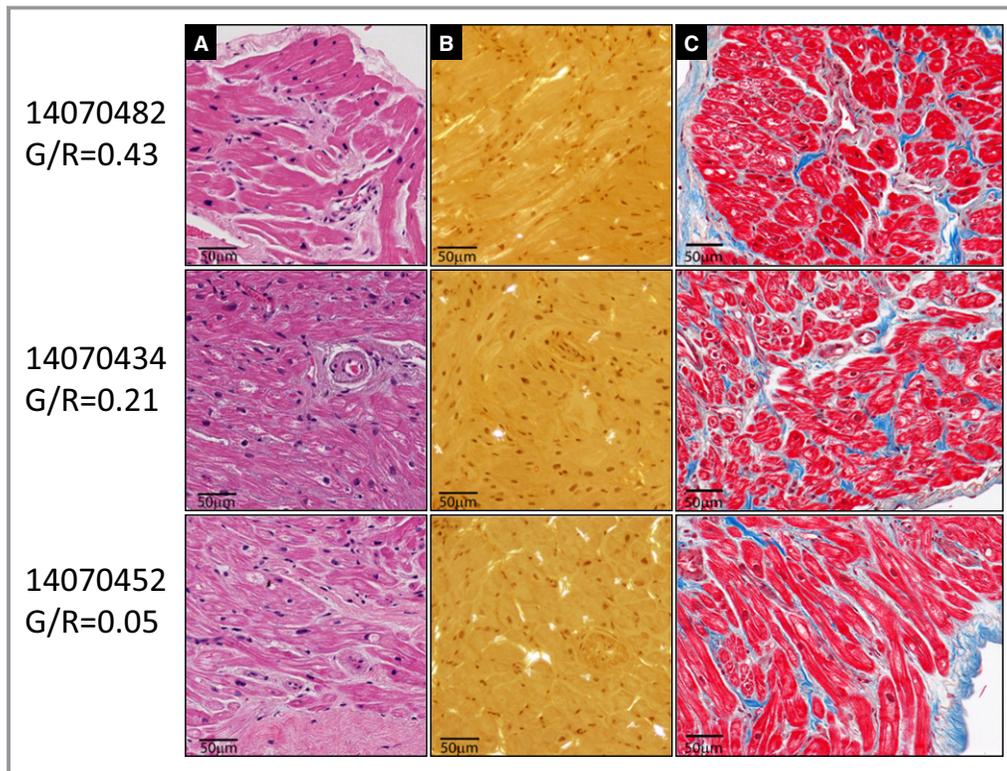
A structural abnormality that can occur in human atrial tissue is fibrosis, resulting from cardiac and systemic comorbidities such as hypertension. To investigate the relationship between PAO burden and fibrosis in atrial tissue, Masson trichrome staining was performed on the samples described above (mean G/R values of 0.03 to 0.43;  $n=29$ ). By general linear model analysis, there was no evidence of a correlation between G/R values and percent fibrosis ( $r=-0.356$ ,  $P=0.5$ , with images illustrated in Figure 3C). For this group of samples, the amount of fibrosis averaged  $16±9\%$ .

### Protein Composition of PAOs

Previous studies in patients with isolated atrial amyloidosis have identified ANP as a uniform component of amyloid plaques (100%) and occasionally TTR (10%). To test the hypothesis that 1 or both of these amyloidogenic proteins are present in atrial PAO formation, adjacent sections were immunostained for PAOs and for either ANP or TTR. As shown in Figure 4, there was extensive colocalization of PAOs with ANP in atrial myocytes, whereas Figure 5 illustrates that TTR was found primarily in interstitial regions, with little detectable signal for TTR in the myocytes themselves and minimal colocalization with PAOs. These findings indicate that ANP is a significant component of PAOs in human atrium, while TTR is not.

### Association of PAO Burden With Hypertension

To investigate the possible association of PAO burden with clinical variables of interest, we used a linear mixed-effects model to examine age, sex, hypertension, body mass index, coronary artery disease, type of cardiac disease, tissue source (RA versus LA), and preoperative β-blocker therapy. This analysis included 535 individual field G/R values obtained from the 92 samples. As shown in Table 2, a consistent finding across multiple analyses was the independent association of PAO burden with the presence of hypertension ( $P=0.010$ ). This covariate, hypertension, remained significant when controlling for the predominant type of cardiac disease in the patient population (ie, AV replacement). In Figure 2, data for hypertensive patients are highlighted in red. For higher G/R values (ie,  $≥0.13$ ), the prevalence of hypertension was 92% (24 of 26 samples). In this cohort of patients, our analysis did not identify an association of PAO burden with the development of postoperative AF.



**Figure 3.** Representative histologic features in atrial samples from patients with low, medium, and high green/red ratio (G/R) values. For each patient sample (rows), the columns from left to right display images showing: hematoxylin and eosin–stained section (A); Congo red–stained section with visualization using polarized light (B); and Masson’s trichrome–stained section demonstrating collagen (blue) representing areas of fibrosis (C). The G/R values are shown to the left of the images.

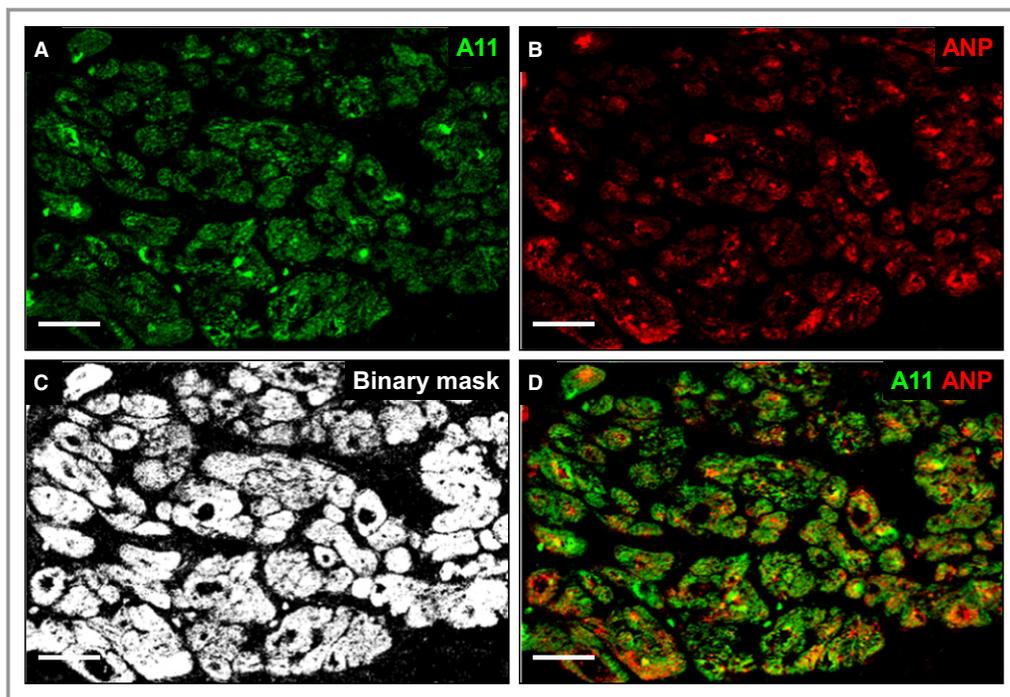
## Discussion

Our results are the first to demonstrate that PAOs are present in human atrium, as prior experimental and clinical studies have focused exclusively on ventricular tissue.<sup>16,17,26,27</sup> Abnormal protein aggregation is being increasingly linked to degenerative diseases caused by aging.<sup>7,9</sup> Given that a senile form of amyloidosis can occur exclusively in the atrium, it is not surprising that PAOs would develop there as well. However, the prevalence and significance of atrial PAOs have not been previously characterized. When controlling for multiple clinical variables in this patient population, PAO burden was consistently associated with the presence of clinical hypertension.

Atrial arrhythmia susceptibility in the presence of hypertension remains poorly understood. Both hypertension-induced fibrosis and cardiac chamber enlargement can create the substrate for reentry and thus AF. However, a sizable proportion of patients with hypertension lack both features, and the arrhythmogenic mechanism(s) in these patients remains unclear. It is intriguing to speculate that PAOs may form a missing link between hypertension and atrial pathophysiology/arrhythmias. However, future studies in patients

with established AF are required to further investigate this hypothesis. While our analysis of this relatively small cohort did not identify a link between PAOs and postoperative AF, this type of AF is linked to the inflammatory response associated with cardiac surgery, and it can occur regardless of a prior history of AF and/or risk factors.

In organ-specific amyloidosis such as Alzheimer disease, PAOs correlate most closely with disease phenotype, rather than amyloid deposits.<sup>9,10</sup> As demonstrated in the brain, PAOs can be associated with organ dysfunction in the absence of demonstrable cellular pathology,<sup>10,28</sup> and their capacity to promote injury of cells, including cardiomyocytes, is well documented.<sup>7,10</sup> In neurons, the mechanisms of PAO-related cytotoxicity, or proteotoxicity, are myriad, and they include disturbances in protein clearance, endoplasmic reticulum stress, mitochondrial dysfunction with increased production of reactive oxygen species, increased intracellular calcium, and possible pore formation in the plasma membrane.<sup>5–7,10</sup> In addition to studies of desmin-related cardiomyopathy, data from the Robbins laboratory have demonstrated that cardiac expression of unrelated polyglutamine oligomers causes cardiomyopathy and heart failure, further confirming the cardiac cytotoxicity of PAOs.<sup>27</sup> For ventricular myocytes,



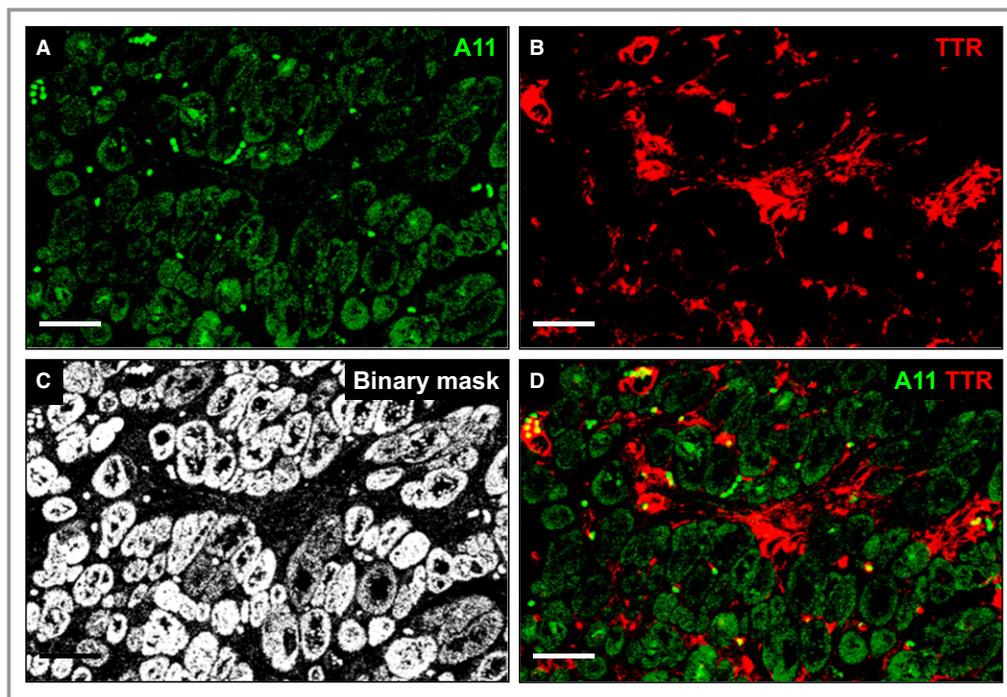
**Figure 4.** Colocalization of ANP and PAO immunoreactivity. Immunofluorescent labeling with PAO-specific (A-11; Panel A) and ANP-specific (B) polyclonal antibodies in adjacent 5- $\mu\text{m}$  human atrial tissue samples. C, Binary mask, representing the area of atrial myocardium. D, Colocalization of A-11 (green) and ANP (red) signals within the myocardium in adjacent sections, as evidenced by the lighter green and yellow regions, compared with (A). Scale bars=50  $\mu\text{m}$ . ANP indicates atrial natriuretic peptide; PAO, preamyloid oligomers.

mechanisms of PAO-mediated cellular injury include disruption of mitochondrial function, reactive oxygen species regulation, cellular metabolism, and cardiomyocyte mechanics.<sup>11</sup> Further studies in model systems are needed to confirm whether similar mechanisms are operative for atrial myocytes.

Based on the reported composition of amyloid plaques in patients with isolated atrial amyloidosis, we performed immunohistochemistry for the candidate proteins ANP and TTR. There was considerable overlap of ANP immunoreactivity in myocytes with that for PAOs, indicating that ANP is a major component of these oligomers. On the other hand, TTR was not colocalized with PAOs but, rather, was predominantly found in interstitial regions of the myocardium. It has long been recognized that ANP is fibrillogenic (ie, it can form protein oligomers and fibrils at high concentrations).<sup>29</sup> There is increasing evidence for mechanistic common ground between hypertension and AF with respect to ANP.<sup>30</sup> Plasma concentrations of both N-terminal pro-ANP and ANP are increased in essential hypertension,<sup>31–37</sup> a finding largely thought to represent a compensatory response to elevated intravascular pressure. Thus, the interaction of these biomarkers and hypertension is biologically plausible and concordant with data from epidemiologic studies. We hypothesize that increased plasma ANP concentrations in the atria during hypertension, as well as local ANP elevations related to atrial

stretch, are sufficient to promote atrial PAO formation due to ANP aggregation (Figure 6).

The distribution of oligomers was nonhomogeneous throughout the atrial myocardium, in a pattern reminiscent of that seen with atrial fibrosis.<sup>3,38</sup> Data from previous studies indicate that the presence of isolated atrial amyloid is linked with an increased risk of AF (Figure 6). Like fibrosis, amyloidosis can cause local conduction block and electrical heterogeneity that would promote reentrant circuits and therefore AF. In a study of 245 patients undergoing cardiac surgery,<sup>12</sup> Congo red staining was identified in interstitial and endocardial deposits in 40 patients (16.3%). Amyloid deposits were immunoreactive for ANP in all patients, and for TTR in 4 (10%) cases. By univariate analysis, atrial amyloid was found more commonly in patients with AF. In a report of 72 patients with rheumatic valvular disease and chronic AF,<sup>39</sup> amyloid deposits were detected in the atria of 33 patients (46%), compared with 6 of 52 patients (12%) with severe heart failure in sinus rhythm. In addition, the presence of atrial amyloid correlated with an increased duration of AF. In autopsy samples from 100 elderly patients (mean age of 75 years),<sup>13</sup> intra-atrial amyloid was detected in 80 hearts (all with ANP immunoreactivity). In general, deposits were more intense in patients with chronic AF. While these studies suggest an association between atrial amyloid and AF, the majority of



**Figure 5.** Lack of colocalization for TTR and PAO. Immunofluorescent labeling with PAO-specific (A-11; A) and TTR-specific (B) polyclonal antibodies (TTR) in adjacent 5  $\mu\text{m}$  human atrial tissue samples. C, Binary mask, representing the area of myocardium. D, Resulting merged image of A-11 (green) and TTR (red) signals in adjacent sections, demonstrating minimal evidence of colocalization. Scale bars=50  $\mu\text{m}$ . PAO indicates preamyloid oligomers; TTR, transport protein transthyretin.

**Table 2.** Mixed-Effects Model for the 92 Patients

	Estimate	SE	P Value
<b>Fixed effects</b>			
Intercept	−3.483	0.749	0.000**
Hypertension	0.452	0.172	0.010*
Sex	−0.055	0.155	0.725
Age	0.005	0.006	0.377
BMI	0.009	0.015	0.532
$\beta$ -Blocker use	−0.099	0.161	0.540
AV replacement	−0.506	0.369	0.074
Atrial source	0.218	0.398	0.585
<b>Random effects</b>			
Intercept	0.676		

AV indicates aortic valve; BMI, body mass index.

\* $P < 0.05$ ; \*\* $P < 0.01$ .

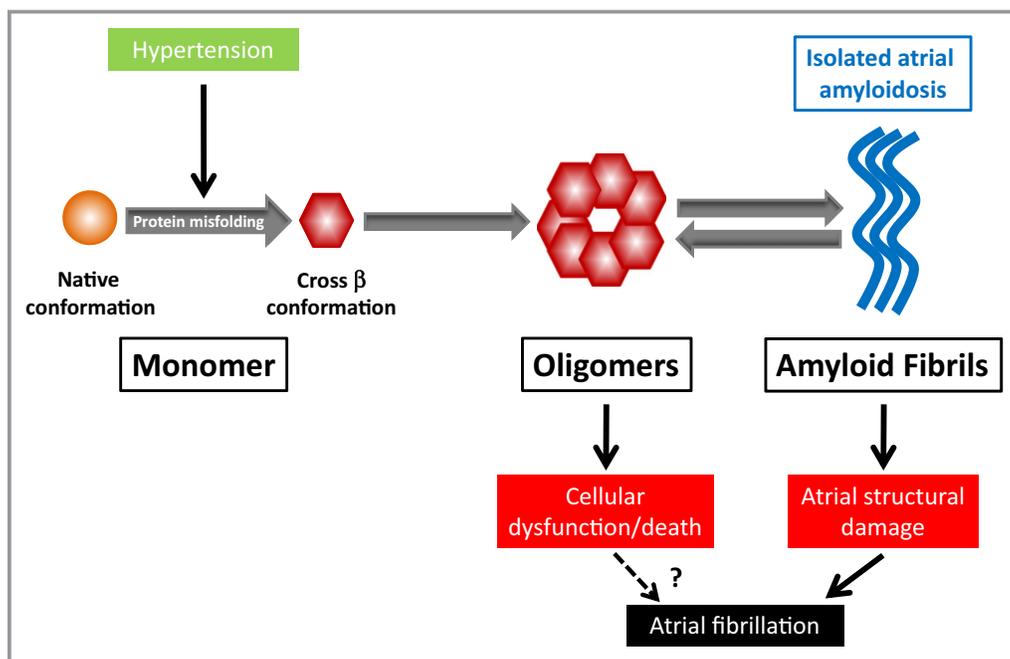
patients with AF do not demonstrate Congo red–staining atrial deposits. As noted here earlier, future investigations to compare PAO burden in the atrial tissue of patients with and without established AF will be critical.

Based on existing data, protein misfolding may represent a novel molecular target in the treatment of cardiac disease.<sup>7,10,11</sup> The most common hereditary systemic amyloido-

sis is caused by mutations in the gene encoding TTR, with multiorgan involvement.<sup>8,40,41</sup> Accumulation of misfolded wild-type TTR can also cause senile amyloidosis. However, this differs from isolated atrial amyloidosis in that its distribution is systemic, and it typically involves the vasculature and myocardial interstitium.<sup>40,41</sup> Clinical trials are currently under way to investigate the therapeutic utility of targeting protein misfolding (eg, agents that promote TTR stabilization or alter the formation/catabolism of amyloid species [ie, doxycycline] to treat systemic amyloid-related diseases).<sup>7,10,11</sup>

### Limitations

The limitations of this study include the small size of the atrial samples, the heterogeneous nature of the cardiac disease substrate for the patients studied, and the fact that both LA and RA samples are included for analysis. Nonetheless, it is reassuring that despite these limitations, we demonstrated that PAO burden was similar for LA and RA, and the association of PAO burden with hypertension was significant when controlling for major clinical factors (including atrial source) in our analysis. Given that atrial samples were collected during cardiac surgery, the atrial sample size is limited. This likely accounts for the fact that Congo red–positive



**Figure 6.** Hypothesis linking hypertension, PAO formation, and atrial pathology/arrhythmogenesis, compared with the known process of isolated atrial amyloidosis. In the presence of hypertension, protein misfolding and oligomerization can occur in the atrium, with ANP a principal component of PAOs. Protein oligomers can directly cause myocyte injury/death, or they can coalesce with aging to form amyloid fibril deposition in atrial myocardium. This process, known as isolated atrial amyloidosis, is associated with infiltrative structural damage in the atrium and an increased risk of atrial fibrillation. ANP indicates atrial natriuretic peptide; PAO, preamyloid oligomers.

amyloid deposits were not detected in the 16 specimens studied, given this abnormality is more likely to be detected if multiple atrial samples are examined.<sup>42</sup> Also, ventricular samples were not obtained during cardiac surgery to allow for comparison with atrium. The patients in this study were almost exclusively white; therefore, it is not clear if these results can be extrapolated to other ethnic patient populations. An additional limitation is the number of patients in the cohort and the prevalence of hypertension, factors that at least to some extent reduce the power of our findings. Data regarding the severity of hypertension, cardiac hemodynamics, and cardiac structural parameters for individual patients were also not available. Further studies would be useful to further confirm the association between atrial PAO burden and hypertension in a larger cohort of patients that includes those with nonvalvular cardiac pathology (eg, coronary artery disease). Finally, it is possible that the atrial PAOs detected in this study are composed of other fibrillogenic proteins besides ANP.

## Conclusions

Our results demonstrate evidence for a novel potential mechanism (ie, cytotoxic PAOs), whereby hypertension can

promote atrial pathology. Future studies to examine the relationship of atrial PAO burden to various forms of AF will be of considerable interest.

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## Disclosures

None.

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