October 28, 2015

Dear Editor,

Please find enclosed the edited manuscript in Word format (file name: 22768-Revised manuscript.doc).

Title: Bicuspid Aortic Valve Hemodynamics Does Not Promote Remodeling in Porcine Aortic Wall Concavity

Authors: Samantha K. Atkins, Alison N. Moore, Philippe Sucosky

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The manuscript has been improved according to the suggestions of the reviewer. We would like to thank the positive and constructive comments provided by the reviewers on our manuscript. This document includes the authors' response to the comments as well as a summary of the changes made to the revised manuscript. We feel that the revisions and additions we have made strengthen the original data and the overall quality of the paper.

1. **It would have been reasonable to collect the data obtained in aortic concavity and convexity in a single paper, and not refer repeatedly to the previously published paper. This would also have allowed a direct comparison of expression and activity of MMP-2 and MMP-9 between concavity and convexity of the same animals, submitted to a TAV or BAV-like wall shear stress.**

   As suggested by the reviewer, the use of aorta convexity and concavity tissue specimens excised from the same animal combined with the ex vivo technique described in our study would be the ideal methodology to characterize the differential effects of convexity and concavity hemodynamics on the biology of the aortic wall. However, we would like to point out two important considerations. First, the ex vivo tissue culture technique described in our paper is currently the only methodology capable of isolating the role played by hemodynamic forces in BAV aortopathy while effectively discarding the effects of other surrounding genetic and biochemical factors. Second, for the study to be of significance, the experiments need to be conducted on tissue that is as close as possible as its native counterpart biochemically, biologically and structurally. Based on our experience with the shear stress bioreactor, the experimental protocol necessary to prepare six specimens, mount them in the bioreactor, condition them to shear stress, harvest them from the bioreactor, and prepare the bioreactor for the next run takes three days and a half. Therefore, should the experiments be conducted using tissue from the same animal, some tissue samples would have to be stored in sterile PBS and exposed to static conditions over that duration. We and other investigators have demonstrated that such conditions are detrimental to cardiovascular tissue as they promote cellular apoptosis and loss of mechanical integrity[1-3]. In particular in the context of AA tissue, we have already

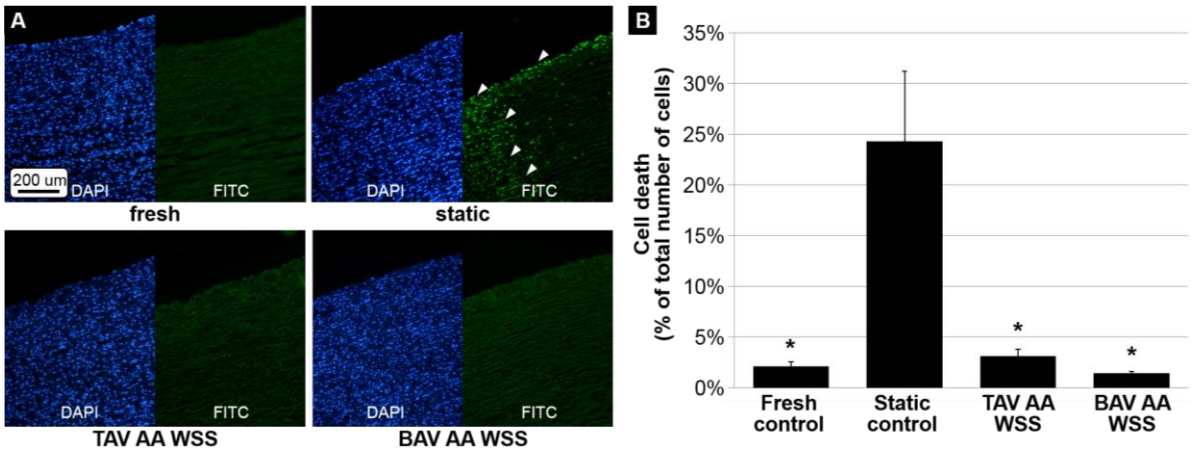

Figure R1: *Cellular apoptosis in fresh tissue specimens excised from the AA convexity, tissue statically incubated in the shear stress bioreactor for 48 hours and tissue exposed to convexity TAV AA WSS and BAV AA WSS for 48 hours: (A) TUNEL assay (green: apoptotic cells, blue: cell nuclei); and (B) quantitative TUNEL results (*p<0.05 vs. static). Adapted from* [2].

demonstrated the inability of static conditions to maintain AA tissue homeostasis (**Fig. R1**). Nevertheless, we recognize that, ideally, the use of two bioreactors and two incubators would enable to conduct experiments in parallel, which would relieve this undesired outcome. Unfortunately, our equipment does not allow such mode of operation, which is why we conducted experiments on the convexity and concavity in two different studies. We have added this brief justification at the end of the discussion.

2. **Also, the application of the findings to a clinical setting could be quite limited, as several other factors, including local pressure and stretch, are not reproduced by the bioreactor.**
We thank the reviewer and acknowledge the importance of this observation. While we agree that other mechanical forces such as pressure and stretch are important regulators of vascular homeostasis, the consideration of these influences on aortic tissue were omitted in this study in order to isolate the direct relationship between wall shear stress and biological remodeling. In addition, prior to designing our tissue experiments, we conducted a thorough mechanical characterization of the aortic wall downstream of a TAV and a BAV. Using the fluid structure interaction model described in the present study and in our previous study[2], we were able to quantify differences in pressure and circumferential stretch (i.e., dominant stretch component) between the TAV AA and the BAV AA. The pressure and circumferential stretch were captured over the same rectangular region over which the WSS data were obtained. This analysis was conducted in both the convexity and concavity. Regardless of the wall region (i.e., convexity/concavity), the comparison of the pressure and circumferential stretch captured in both AA models revealed the negligible impact of the valve anatomy on those two metrics (**Fig. R2, Table RI**). This mechanical similarity is demonstrated by the absence of substantial differences in circumferential stretch and pressure between the TAV AA and BAV AA (average pressure difference: 0.4% in the convexity and 0.5% in the concavity; average stretch difference: 0% in the convexity and 0.3% in the concavity). The existence of nearly similar stretch and pressure environments on TAV and BAV AAs eliminates the possible involvement of these mechanical signals in the contrasted remodeling activities typically observed between these anatomies.

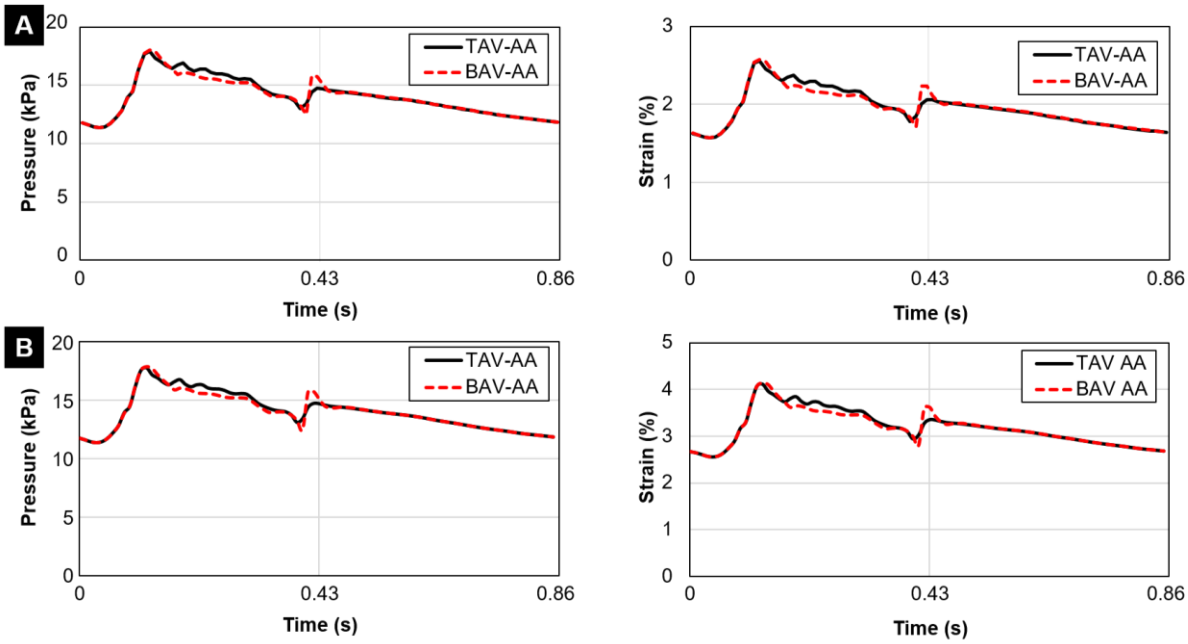

Figure R2: *Pressure and stretch waveforms captured by the computational model in: (A) the convexity; and (B) the concavity of the TAV AA and BAV AA.*

		TAV AA		BAV AA	
		Convexity	Concavity	Convexity	Concavity
WSS	Max (Pa)	2.6	3.2	3.2	3.3
	TSM (Pa)	0.49	0.77	0.95	1.06
	OSI	0.42	0.49	0.00	0.18
Pressure	Min	11.37	11.37	11.37	11.37
(kPa)	Max	17.92	17.76	18.01	17.85
	Average	14.04	14.02	13.98	13.95
Stretch	Min	1.57	2.55	1.57	2.55
(%)	Max	2.55	4.12	2.57	4.14
	Average	1.95	3.18	1.95	3.17

Table RI: *Characteristics of the WSS, pressure and circumferential stretch waveforms predicted computationally in the concavity and convexity of the TAV AA and BAV AA.*

On the other hand, the evidence of contrasted WSS characteristics in TAV and BAV AAs described in the present paper (see **Table I** in manuscript and **Table RI** in this response) motivates and justifies the investigation of their potential mechanobiological impact. While those WSS differences were more pronounced in the disease-prone convexity (TSM difference: 94%), they were more subtle in the concavity (TSM difference: 38%) but different enough to warrant an investigation into their role on tissue remodeling. In fact, while the concavity WSS waveforms captured in both anatomies exhibited similar peak values, they were marked by substantially different temporal shear magnitudes (TSM) and oscillatory shear indices (OSI) (**Table RI**).

Lastly, we would like to point out that despite the absence of stretch and pressure in our previous convexity study, tissue specimens exposed to their native WSS exhibited the same structure and same level of cellular apoptosis as those measured in fresh controls. While this

observation does not preclude the possible involvement of stretch and pressure stimuli in other biological processes, it suggests the dominant role played by WSS in vascular homeostasis, even in the absence of the full spectrum of mechanical forces normally found in the native environment. For all those reasons, our focus on WSS mechanobiology seems reasonably justified. We have added this brief justification at the end of the discussion.

3. **Another main concern is the limited number of samples used for each test and experimental group (n=3, statistics paragraph, methods section), especially considering the high standard deviation obtained and represented in graphs in fig. 2 and 3.**

We agree with the reviewer and acknowledge that the study would benefit from a larger sample size. However, we believe that the data reported in our paper still provides new important insights into the mechanisms involved in BAV aortopathy.

First, the sample size considered in the present study (N=3) is similar to that of our previous study on the effects of BAV flow on the remodeling of the AA convexity, in which three samples were used for western blot and three other samples were used for immunohistochemistry[2]. Since western blot analysis was not performed in the present study, the use of three tissue specimens is consistent with the methodology of our previous study.

Second, the use of three samples for the generation of all quantitative results in our previous study was able to demonstrate statistically significant biological differences between convexity specimens subjected to TAV AA and BAV AA WSS. In this context, the absence of statistical differences in the remodeling response of concavity tissue samples subjected to TAV AA and BAV AA flow is likely to be a reflection of the low impact of the concavity WSS environment on the local tissue biology rather than the consequence of a small sample size.

Third, the absence of significant tissue remodeling in concavity tissue suggested in the present study is in agreement with the data reported in larger clinical studies that examined the asymmetric nature of aortic dilation and the spatiotemporal patterns of MMP expression in BAV AAs[4–8].

Therefore, while a larger sample size would increase the confidence level, it is not expected to generate any statistical difference between the experimental groups. The combination of the present results, our previous convexity data and previous clinical reports strengthens the support for the existence of hemodynamic mechanisms in BAV aortopathy and the particular sensitivity of BAV AA to its regional WSS environment. We have added this justification in the Materials and Methods (Statistical Analyses section) and at the end of the discussion.

4. **The use of confocal microscopy could allow a more quantitative analysis of MMP-2 and MMP-9 expression in aortic cross-sections.**

The first step in examining the causality between wall shear stress abnormities on the asymmetric presentation of ascending aortic dilation requires a standardized methodology capable of isolating, replicating and analyzing local differences in the biological signature of intact aortic tissue. While we agree with the reviewer that alternate imaging techniques may allow for a more quantitative analysis of MMP expression, it was critical to maintain consistency in the assessment methods between the previous and present studies. The implementation of the same methodology allowed for the direct comparison of the biological results obtained in the convexity and concavity of the aortic wall. In addition, while confocal microscopy is ideal for higher magnification applications and for examining protein expression at the cellular level, it may not be suitable to quantify the sparse expression of MMPs observed throughout the entire

aortic wall medial layer. In this context, although confocal microscopy could nicely complement our study, we feel that immunostaining was more appropriate for the assessment of the tissue remodeling state.

Reference cited in this response:

1. Balachandran K, Konduri S, Sucosky P, Jo H, Yoganathan AP. An ex vivo study of the biological properties of porcine aortic valves in response to circumferential cyclic stretch. *Ann Biomed Eng* 2006;**34**:1655–65 [PMID: 17031600 DOI: 10.1007/s10439-006-9167-8]

2. Atkins SK, Cao K, Rajamannan NM, Sucosky P. Bicuspid Aortic Valve Hemodynamics Induces Abnormal Medial Remodeling in the Convexity of Porcine Ascending Aortas. *Biomech Model Mechanobiol* 2014;**13**:1209–25 [PMID: 24599392 DOI: 10.1007/s10237-014-0567-7]

3. Levesque MJ, Nerem RM. The elongation and orientation of cultured endothelial cells in response to shear stress. *J Biomech Eng* 1985;**107**:341–7

4. Della Corte A, Quarto C, Bancone C, Castaldo C, Di Meglio F, Nurzynska D, De Santo LS, De Feo M, Scardone M, Montagnani S, Cotrufo M. Spatiotemporal patterns of smooth muscle cell changes in ascending aortic dilatation with bicuspid and tricuspid aortic valve stenosis: focus on cell-matrix signaling. *J Thorac Cardiovasc Surg* 2008;**135**:8–18 [PMID: 18179910 DOI: 10.1016/j.jtcvs.2007.09.009]

5. Della Corte A, Romano G, Tizzano F, Amarelli C, De Santo LS, De Feo M, Scardone M, Dialetto G, Covino FE, Cotrufo M. Echocardiographic anatomy of ascending aorta dilatation: correlations with aortic valve morphology and function. *Int J Cardiol* 2006;**113**:320–6 [PMID: 16413075 DOI: 10.1016/j.ijcard.2005.11.043]

6. Ikonomidis JS, Jones JA, Barbour JR, Stroud RE, Clark LL, Kaplan BS, Zeeshan A, Bavaria JE, Gorman JH, Spinale FG, Gorman RC. Expression of matrix metalloproteinases and endogenous inhibitors within ascending aortic aneurysms of patients with bicuspid or tricuspid aortic valves. *J Thorac Cardiovasc Surg* 2007;**133**:1028–36 [PMID: 17382648 DOI: 10.1016/j.jtcvs.2006.10.083]

7. Lu MT, Thadani SR, Hope MD. Quantitative assessment of asymmetric aortic dilation with valve-related aortic disease. *Acad Radiol* 2013;**20**:10–5 [PMID: 22951111 DOI: 10.1016/j.acra.2012.07.012]

8. Cotrufo M, Della Corte A, De Santo LS, Quarto C, De Feo M, Romano G, Amarelli C, Scardone M, Di Meglio F, Guerra G, Scarano M, Vitale S, Castaldo C, Montagnani S. Different patterns of extracellular matrix protein expression in the convexity and the concavity of the dilated aorta with bicuspid aortic valve: preliminary results. *J Thorac Cardiovasc Surg* 2005;**130**:504–11 [PMID: 16077420 DOI: 10.1016/j.jtcvs.2005.01.016]