Inflammatory Serum Factors from Aortic Valve Stenosis Patients Drive Sex Differences in Valve Myofibroblasts

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Statement of Purpose: Aortic valve stenosis (AVS) is a progressive disease affecting nearly 13% of patients over the age of 75 that causes fibrosis and/or calcification of valve tissue[1]. AVS is sexually dimorphic, with men typically experiencing increased calcification of aortic valve leaflets relative to women, who show increased fibrosis in valve tissue. Furthermore, disparities in the clinical treatment of AVS exist, where women are less likely to be diagnosed with severe AVS[2]. Inflammatory factors present in AVS patient blood serum are thought to contribute to sex differences in AVS progression by driving the activation of valvular interstitial cells (VICs) to myofibroblasts and/or osteoblast-like cells[3], which leads to fibro-calcification of the aortic valve. However, the molecular mechanisms by which inflammatory factors contribute to AVS progression remain largely unknown.

In this study, we collected AVS patient serum from severe AVS patients prior to valve replacement and identified inflammatory serum factors that modulate sex differences in VIC myofibroblast activation and osteoblast-like differentiation. Our strategy was to correlate serum proteome data to myofibroblast activation levels in valvular and cardiac fibroblasts cultured with patient sera *in vitro* and clinical measures of AVS disease severity. We hypothesized that the identified candidate proteins would drive increased myofibroblast activation in female VICs and increased osteoblast-like differentiation in male VICs. To test this hypothesis, candidate factors (specifically, annexin A2, cystatin C, and Dickkopf-related protein 3) were chosen for *in vitro* validation and used to treat male and female VICs cultured on hydrogels engineered to recapitulate the valve extracellular matrix. Sex-specific responses in myofibroblast activation and osteoblast-like differentiation in response to these factors were observed.

Methods: Serum samples were collected from AVS patients and analyzed using a SOMAscan DNA aptamer array as described previously[4]. Significant proteins were identified using an original code developed in R that filtered 1,300 serum proteins that were significantly correlated (R=0.8) with at least one known measure of AVS severity. A similar code was developed and used to identify serum proteins that likely drive myofibroblast activation in VICs by screening for factors significantly correlated (R=0.75) with previous *in vitro* VIC and ventricular fibroblast myofibroblast activation data[5].

VICs were isolated from 5-6 month old porcine aortic valves and seeded onto poly(ethylene glycol) (PEG) hydrogels designed to mimic a healthy aortic valve (elastic modulus ~6 kPa), which were formed using thiol-ene click chemistry as previously described[5]. Male and female VICs were cultured separately, then seeded onto hydrogels for two days with media containing varying concentrations of annexin A2, cystatin C, or Dickkopf-related protein 3. Cells were then fixed, immunostained, and quantified in Matlab, with myofibroblast activation measured through α-SMA gradient mean intensity and osteoblast-like differentiation measured through RUNX2 nuclear localization.

Results: Out of the over 1,300 serum proteins analyzed, we identified 125 clinically significant proteins and 347 proteins linked to *in vitro* myofibroblast activation, resulting in 38 candidate proteins (Fig 1A). As an example, annexin A2 (ANXA2) was one of the 38 candidate proteins, which was significantly correlated with patient Society of Thoracic Surgeons (STS) score (Fig. 1B) and ventricular fibroblast myofibroblast activation (Fig. 1C). ANXA2 increased myofibroblast activation in female VICs relative to male VICs (Fig. 1D). Conversely, ANXA2 drove a significant increase in osteoblast-like differentiation only in male VICs at the highest concentration tested (Fig. 1E).

Conclusions: Our work showcases the importance of (1) sex-separating cells and (2) utilizing bioengineered hydrogels as physiologically relevant cell culture platforms to evaluate sex-specific responses to inflammatory factors. We identified ANXA2 as a clinically significant serum factor that promotes myofibroblast activation more in female VICs and osteoblast-like differentiation only in male VICs. Ongoing work suggests that MAPK signaling modulates sex differences in VIC phneotypes treated with ANXA2. In sum, identifying inflammatory factors that promote sex-specific AVS progression may lead to the development of sex-specific drug treatments.

References: [1] Osnabrugge, RL. J. Am. Coll. Cardiol. 2013, 62:1002–1012. [2] Raddatz, MA. Open Heart. 2020. [3] Grim, JC. Arterioscler. Thromb. Vasc. Biol. 2020, 40:296-308. [4] Aguado, BA. Sci. Transl. Med. 2019, 11. [5] Fairbanks, BD. Adv. Mater. 2009, 21:5005-5010.

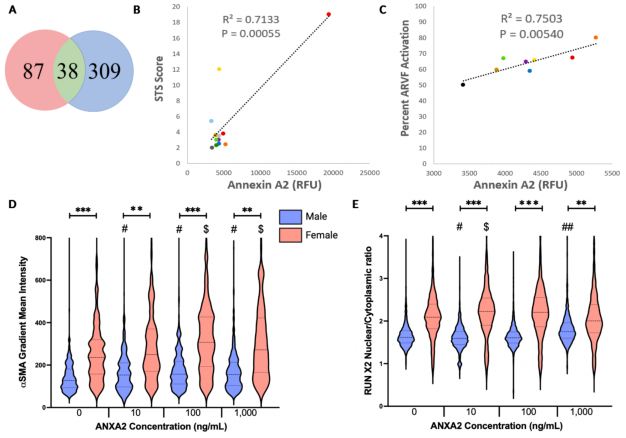

Figure 1: (A) Proteins significantly correlated with at least one measure of AVS severity (red circle) compared with proteins significantly correlated with myofibroblast activation *in vitro* (blue circle). (B) Correlation graph comparing ANXA2 abundance and patient STS score. (C) Correlation graph comparing ANXA2 abundance and ARVF percent activation *in vitro*. (D) Female VICs have increased α-SMA intensity compared to male VICs when cultured with ANXA2. (E) Male VICs show increases in RUNX2 nuclear localization in response to ANXA2 at the highest concentration. Cohen's d-value used for statistical significance with ***d>0.8, **d>0.5, and *d>0.2 for sex differences, $^{\#\#}$d>0.5 and $^{\#}$d>0.2 for differences from male control, and $^{\$}$d>0.2 for differences from female control.