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The Heart of the Matter: How Cellular Sex and Sex Hormones Influence CAVD Pathogenesis

By

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A dissertation submitted in partial fulfilment of

the requirement for the degree of

Doctor of Philosophy

(Biomedical Engineering)

at the

UNIVERSITY OF WISCONSIN MADISON

2024

Date of oral examination: 08/02/2024

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Chapter 8

CHAPTER FOR A GENERAL AUDIENCE

The Wisconsin Initiative for Scientific Literacy is a fantastic initiative with the aim of bringing STEM research to a broader audience and making it more accessible for non-scientists. In a post-COVID world, we are constantly bombarded with information by the media that attempts to distill down complex research for the public. Sometimes the media can get it right, but oftentimes they miss the mark on what the research was actually trying to convey. I hope that through this collaboration with WISL I can break down my motivations for my research, and simplify it for a non-scientific audience. Additionally, I was able to conduct the research I did through publicly funded dollars, and as such I firmly believe that my research should be accessible to the public. Hopefully, reading about my work will encourage people to ask the right questions with regards to the science they see disseminated online and make more informed choices for themselves.

In my nearly 6 years at UW Madison in the Biomedical Engineering department, I have participated in many outreach events. In fact, I was given the exciting opportunity to participate in an Art-Science initiative courtesy of the Marie Kohler Fellowship through the Wisconsin Institute for Discovery. I was paired with a dancer, and we collaborated with a Biomechanics lab on campus to translate dance movements into data using sensor technology in a project titled “Bodies in Motion”. Participating in this project was incredibly fulfilling and allowed me to view science through a creative lens. Not only that, but it also forced me to think about how to

communicate niche concepts to a non-scientific audience. Of course, having a taste of this, I didn't stop there and went on to participate in three-minute thesis style competitions trying to find fun ways to explain what I do in three minutes or less to people who knew nothing about my work. These were some of the motivations that prompted me to write a chapter in my thesis for the general public, so my years of work did not remain constrained to just peers, but could also be communicated to the people this research might affect.

8.1 Allow me to introduce you to your aortic valve

The entirety of my research centers about a very small part of the heart that you likely never think about. If you think of the heart as a transit hub of the body, the aorta would be its biggest highway, and as with any highway, the maintenance of traffic is key to avoiding accidents. In the case of our hearts, the aorta has a valve that ensures blood flows only in one direction **(Figure 8.1).** In case this aortic valve stops working for any reason, blood would flow back into the chambers of the heart and eventually this could lead to heart failure.

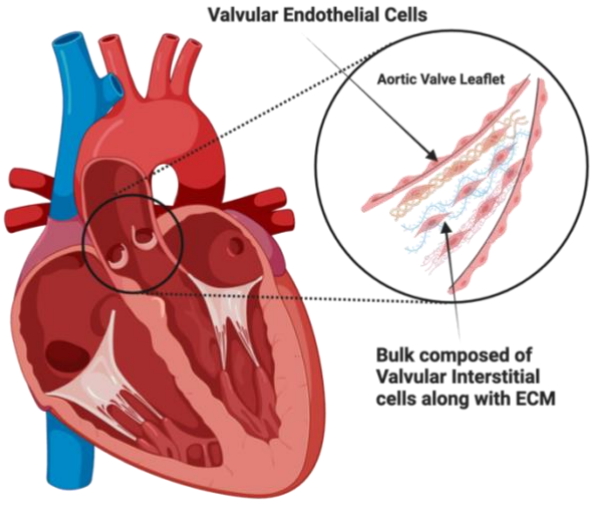

Figure 8.1: Illustration of an anatomical heart depicting the position of the aortic valve.

The aortic valve can function normally when its three leaflets are able to open and close properly with no obstructions. Each leaflet is surprisingly complex for such a thin membrane, and is constantly being remodeled so the leaflets can withstand the blood flow and turbulence. To understand how each leaflet is structured, imagine a sandwich **(Figure 8.2)**.

The proverbial “bread” is the single layer of Valvular Endothelial Cells (VECs) that line the outsides of the leaflet. The “filling” is made up of various extracellular matrix (ECM) molecules. This includes proteins that you may have heard of, like collagen, which is the most abundant valve protein, and gives the leaflets strength to withstand the flow of blood. There are also water-loving molecules in the middle that act as a cushion against shocks to the valve, and contain another well-recognized molecule from many beauty products – hyaluronic acid. Finally, towards the side of the valve facing the ventricular heart chamber, there is a layer of elastin that makes the leaflets flexible.

This ECM acts like a “sauce,” or glue, holding everything together, and is produced by the cells that are the “meat” of the valve. The “meat” is made up of the Valvular Interstitial Cells (VICs) responsible for pumping out ECM molecules in response to many different factors. In a healthy valve, there is a balance of all these different components, making for the perfect sandwich. However, when risk factors are introduced into the equation, this sandwich starts to malfunction with the “filling” overexpanding the leaflet.

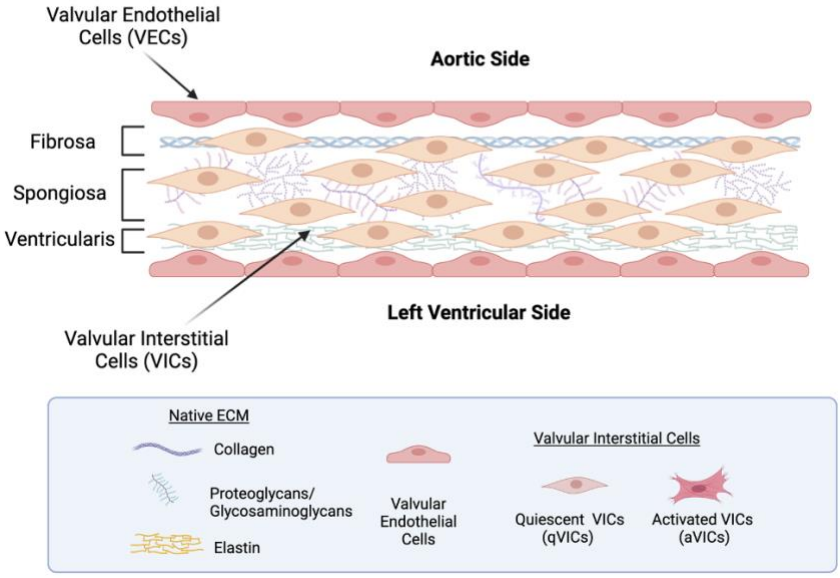

Figure 8.2: The Aortic Valve Sandwich. The Valvular Endothelial Cells (VECs) form a single layer on the outside of the valve. The bulk is composed of an extracellular matrix (ECM) that contains Valvular Interstitial Cells (VICs). These different components help the valve withstand the blood flow and react to different factors that affect heart health.

8.2 What is Calcific Aortic Valve Disease (CAVD) and why should you care about it?

The valve dysfunction I spoke about earlier usually happens in stages **(Figure 8.3),** and is triggered by a variety of factors. The layers of the leaflet are affected in different ways, with overproduction of ECM molecules by the VICs causing the valve to get thicker. Eventually, there is a formation of bone-like deposits in the leaflets – therefore the disease is often called Calcific Aortic Valve Disease, or CAVD. These deposits cause the valve to become narrower, with the valve becoming "leakier," as it cannot open or close fully, making it unable to perform its function properly. The reason why this is worrisome is because doctors are rarely able to detect CAVD in the early stages,

and there is no medical treatment available to slow down or reverse CAVD. What this means for patients is that they are forced to undergo a total aortic valve replacement, and get either a mechanical valve or one sourced from a pig, both of which have a finite lifespan.

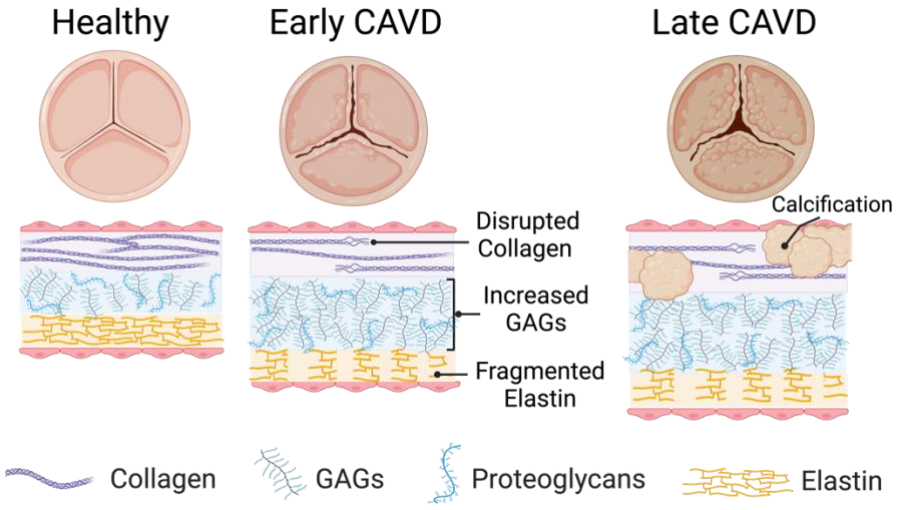

Figure 8.3 The different stages of aortic valve dysfunction with thickening of the valve in the early stages followed by calcification, where bone-like deposits form within the valve. This causes stenosis, or narrowing of the valve, which disrupts its function.

What we do know about the disease is what might trigger the initiation of CAVD. Some of the risk factors for CAVD you may already be aware of: a poor diet, diabetes, aging and smoking can all contribute to increasing your risk of developing aortic valve disease. In addition to those things, there is also evidence that men are twice as likely to develop aortic valve dysfunction.

8.3 Battle of the Sexes

Not only are men at a higher risk for CAVD, but the disease also presents differently in men and women. For the same extent of disease progression, valves from men tend to have bone-like deposits, whereas women tend to have more scar-like tissue, or fibrosis **(Figure 8.4)**. This suggests that biological sex, and here I do mean sex as opposed to gender, may be a contributing factor in how CAVD can develop differently between the sexes. More importantly, we do not actually know the cause for the differences we see between men and women, which was a major reason why I decided to investigate this question.

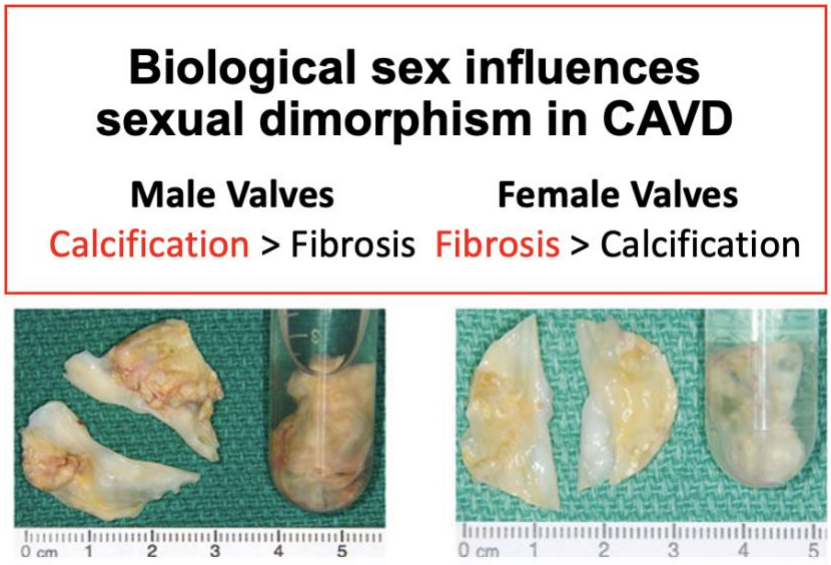

Figure 8.4 Aortic valve leaflets obtained from male and female CAVD patients show different presentations of how the disease develops – men have calcific deposits whereas women have a buildup of scar-like tissue[1].

It was only in 2016 that the National Institutes for Health began to require research to include biological sex as an important factor in all research. In fact, most research was conducted only with male patients, animals and cells. There was a prevalence of outdated notions, such as the

belief that hormonal influences in female animals would lead to variable outcomes, all of which have been debunked. Instead, knowing that male versus female physiology can lead to diverse outcomes in different diseases should be a motivator for researchers to pursue an understanding of how that physiology can affect patient outcomes.

Aside from biological sex, another factor that can influence cell behavior is sex hormones. We know that sex hormones are important in the context of CAVD because the risk for CAVD in women increases after menopause, when there is a decrease in estrogen. There has also been evidence that testosterone might exacerbate CAVD. Contrary to popular belief, men do have some estrogen in their bodies, which is needed for healthy reproductive function. Although, directly treating men with estrogen has unfortunately led to poor outcomes and side effects. That said, I considered both biological sex and estrogen as important factors for CAVD research, especially as I am a woman with a family history of cardiovascular disease.

8.4 The Experiment

So how did I approach this problem? Well for starters, as most research has focused on the late stages of this disease, I was interested in finding out more about the early stages. It is nearly impossible to get healthy valve samples from humans with early stages of disease development, so instead we used pigs. Thanks to Hoesley's Meats, a butcher based in New Glarus who kept us supplied with fresh pig hearts, I was able to isolate fresh VECs and VICs for experiments. As these pigs are butchered before puberty, or they are castrated, they are devoid of hormones that could

interfere with my results. During isolation, I combined the valves from three different pigs for each sex so I could pool together cells from multiple pigs.

To set up this experiment, there were several factors to be considered. I used cells in an "activated" state known as aVICs (activated Valvular Interstitial Cells), which means they are already in a diseased state. I also included a "control" for the estrogen treatment, wherein we cultured cells that receive no estrogen at all. Furthermore, to study the early stages of disease we needed to introduce some sort of stimulus that could push both the VECs and VICs into a further diseased stage, so we introduced TGF-β1 into the equation.

In VICs, TGF-β1 acts as a fibrotic stimulus: it causes VICs to overproduce ECM, which is how the valve becomes thicker and develops scar-like tissue. In VECs (Valvular Endothelial Cells), this stimulus causes the VEC barrier layer to become leakier, because the VECs start to develop into aVICs and separate away from each other. This happens because the VEC monolayer normally exists in a tight cobblestone pattern, once the shape of the cells begins to change, they lose the ability to fit together properly and cannot act like a barrier, thus becoming "leakier". At a molecular level, introduction of a pathological stimulus can cause a domino effect for several reactions within the valve, as depicted in the pictorial summary in **Figure 8.5**.

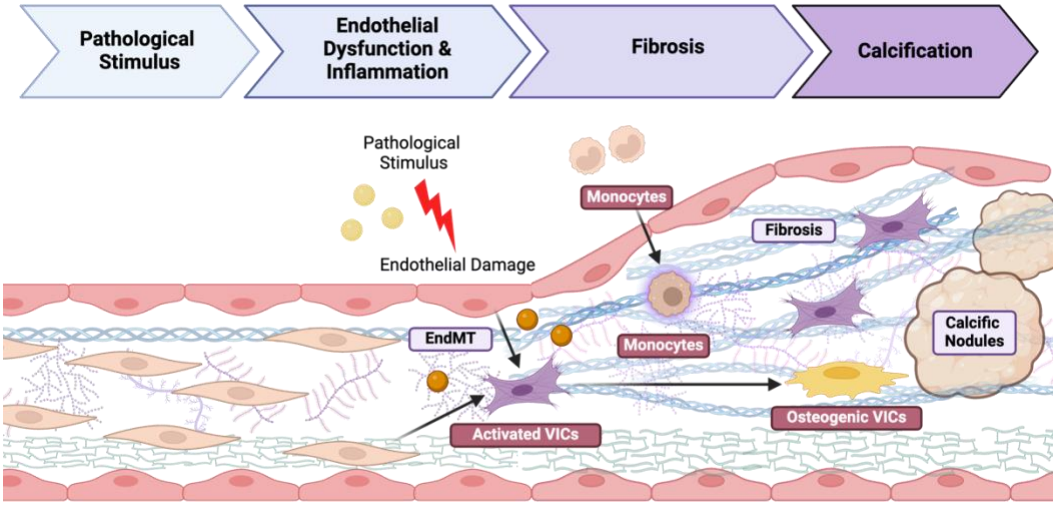

Figure 8.5 Summary of events in CAVD happening at a molecular level within the valve. After introduction of a disease or pathological stimulus, endothelial cells become damaged and transform into aVICs. The healthy VICs also transform into aVICs contributing to valve fibrosis and then later into osteogenic or bone producing VICs that cause calcific deposits.

In the experiment, I cultured cells with many different conditions: the conditions had both male and female cells, with and without estrogen treatment. But also, within these groups, the cells also either did or did not receive TGF-β1. Now that my experimental conditions were established, I wanted to look at different aspects of fibrosis in VICs, the stage where there is a disruption in ECM molecule production. To do this, I first used an assay developed by another lab to tag all newly produced proteins by the VICs with a fluorescent marker. The cells were “fixed,” so they were essentially preserved in the state they were in at the time of fixing for sample processing later on. I also stained the nucleus of the cell, where all the DNA resides, so I could visualize every cell within a field of view.

8.5 The Heart of the Matter

Experiments are conducted in well plates as shown in **Figure 8.6** below, with different configurations of rows and columns so we can test for multiple conditions with their replicates at the same time. I used a 96 well plate for the imaging experiment, with three wells for each condition. After these cells were cultured, I took a total of 9 images per condition for a total of 72 images that were analyzed per plate or experiment. To analyze the fluorescence of the cells per field of view, I used an image analysis software, Image J, for which I wrote code that could quantify area covered by green fluorescence (newly produced protein) and area covered by blue fluorescence (cell nuclei). I then divided the quantification of the green fluorescence with blue, so that I could standardize the amount of newly produced protein to the number of cells present.

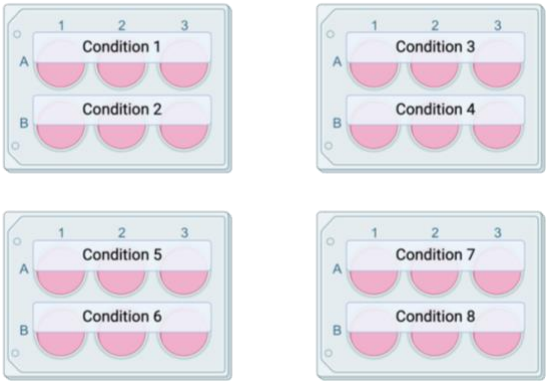

Figure 8.6 Depicts 6-well plates wherein each row represents a condition and has 3 wells representing 3 replicates per condition.

As you can see in **Figure 8.7**, there is a cloud of fluorescent green surrounding blue ovals, which represents the ECM proteins made by the cells enveloping the cells themselves. The results were very interesting because they indicated that estrogen treatment in both males and females was

able to reduce the overall production of new protein. But estrogen treatment also reduced the impact of the fibrotic stimulus that we treated cells with.

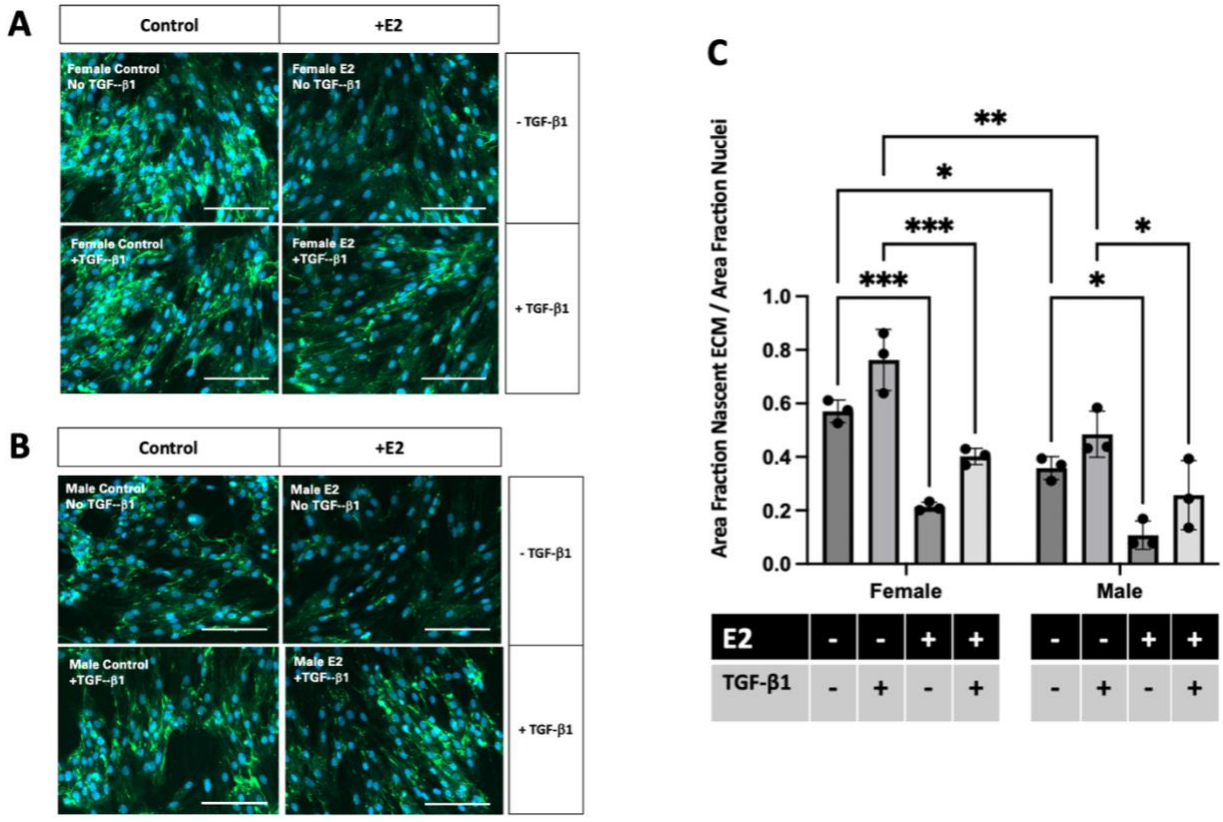

Figure 8.7 Photomicrographs of newly produced proteins in female VICs (8.7A) and male VICs (8.7B) with and without estrogen (E2) or TGF-β1. Although the decrease in new proteins is evident in the images with estrogen treatment, quantification of protein production normalized to cell nuclei confirms the same in 8.7C.

Now that I knew that estrogen had an interesting effect on the ECM proteins being fabricated by VICs, I also used similar methods to image and quantify collagen and fibronectin. I supported it with other analytical methods quantifying different forms of collagen and fibronectin as well. Results followed trends seen in **Figure 8.7** to some extent. Curiously, the trend of estrogen reducing ECM protein quantities and mitigating the effect of TGF-β1 continued to be observed,

but primarily in females only. This means that estrogen is lowering the effect of the disease stimulus on these cells but mainly in females, which suggests that estrogen alone isn't responsible, but that the inherent sex of the cells could be amplifying the effect of estrogen.

Consequently, I went on to examine whether this estrogen affects the signaling pathways of TGF-β1. If your heart is a system of roads, your cells are individual buildings. Inside these buildings, signaling pathways act as communication systems. Each specific molecule, like TGF-β1, is a message for the cell. These messages are delivered to receptors on the surface of a cell, which then pass the message into other molecules within the cell. These can then go to the command center of the cell and give instructions to increase, decrease or keep something the same.

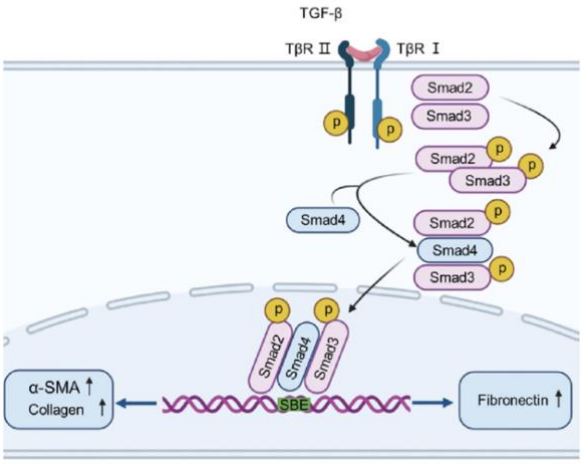

Figure 8.8 Smad communication system within the cells that can be triggered by TGF-β1 and go on to influence cell behavior.

In the TGF-β1 system, Smad proteins are activated by the TGF-β1 receptor, and these proteins get "turned on" or activated via phosphorylation (**Figure 8.8**). I used an assay that tags protein with antibodies to quantify how much normal versus activated Smad protein was being produced. I

found that estrogen once again acted protectively in female VICs, causing them to produce less of the activated Smad proteins. As we saw previously with production of new proteins, once again we notice that estrogen is reducing disease related effects in female cells, which prompted me to think about how sex and estrogen might both be working together to reduce the disease progression in females. These are some of the factors that might be contributing to the lower risk for CAVD in women, along with why we see more advanced stages with bone-like deposits in the valves of men with CAVD.

8.6 What should we take away from this?

There are many different components to my research diving into the different ways estrogen and biological sex can influence disease progression in the aortic valve. The work done by my lab is some of the first to ever consider these factors when studying CAVD. My work is important in filling a knowledge gap within literature, and establishing that both sex and sex hormones greatly impact aortic valve dysfunction. I intended for this research to be a call for action to not only study these sex differences more, but also, knowing that molecular mechanisms can vary so much, we need to develop sex-specific treatments. It is a disservice to people of all sexes to treat them with a one size fits all approach, and instead we should consider experiments and clinical approaches that are inclusive of everyone where possible.

8.7 References

1. Simard L, Côté N, Dagenais F, et al. Sex-Related Discordance Between Aortic Valve Calcification and Hemodynamic Severity of Aortic Stenosis: Is Valvular Fibrosis the Explanation? *Circ Res*. 2017;120(4):681-691. doi:10.1161/CIRCRESAHA.116.309306