DR. MAURICE SARANO: Good morning I'm Dr. Maurice Sarano from the Mayo Clinic, Rochester, and I am here with Dr. Jordan Miller. And today we're going to be talking about aortic valve stenosis and calcification. And as you can see on these images of valve that have stenosis, they are formed by having the calcified cusps that are not with commissural fusion, but are impeded in their movement by the calcium. And today we'd like to discuss the role of calcification in that disease that is frequent in the US.

The classic way of evaluating aortic stenosis is through Doppler using the Bernoulli equation to measure the gradient. And here is an example where the gradient by catheterization and by Doppler are obtained simultaneously, and are almost identical describing the value of this method in assessing the hemodynamics of the aortic valve. We also measure the aortic valve area where you see that the orifice is the ratio of the measurement of the LVOT area multiplied by the LVOT time velocity integral, which is, as a whole, the stroke volume in the LVOT divided by the TVI, Time Velocity Integral, of the aortic jet. These two measures give us a noninvasive way of accessing aortic stenosis.

But what do we know from these measure about the clinical outcome of aortic stenosis? And we're going to show you two studies. One study published from Europe showing that the survival of severe aortic stenosis is excellent in comparison to the general population. One study from the US showing that the survival without AVR is terrible. And these have been questioning the disease as being benign or being malignant. And so there is a doubt on the exact natural history of aortic stenosis.

And a few years ago, the team here at Mayo published this paper on the outcome of more than 600 patients with aortic stenosis, and as you can see, the patients on the left graph have an outcome slightly different from the expected if we stub the follow up when the symptoms occur. If we follow the patient throughout, irrespective of the occurrence of the symptom on the right side, you can see that the patients have an excess mortality that is observed in these cases and in this patient. And so we have this study, which is the largest showing the outcome of aortic stenosis. But there is a discordance between these studies that is difficult to resolve.

And this is why we conducted the study in the community to see the outcome without the referral bias of being referred to a big center. And in those patients you can see that survival after the diagnosis of aortic stenosis, that the best predictor is the aortic valve area below 1 centimeter square. And the reason why this measure is the best predictor of outcome is because some patients have a low gradient and do poorly during the outcome. So indeed, patients with aortic stenosis in the community do poorly when the aortic stenosis is severe.

But this is true also for the patient with low gradient. In that study of patients with low flow, low gradient, low ejection fraction, you can see that the people who underwent surgery had a much better outcome than the people who were left with medical treatment. So that in general, all the studies that we see suggests that when the valve is bad, and we'll see what this means, the outcome is bad and we should consider aortic valve replacement irrespective of the gradient.

So the next issue is how do patients present in the community? And in that study in the community, what we saw is that in Olmsted County, the mean valve area diagnosis is 1.2 centimeters squared with the low gradient of 22 millimeter of mercury. And even in those patients without deadly comorbidity of 74 years old, the diagnosis was late at an advanced stage in the middle of the seventh decade. And in most patients, even those severe in the red column, one can see that whether it's the velocity or the mean gradient, the threshold reached by these patients with so-called severe aortic stenosis, is that below the thresholds that are currently given by the guidelines in terms of definition of severe aortic stenosis, less than 4 meters per second for velocity, less than 40 millimeters of mercury for mean gradient. So that it is frequent in the community to observe patients with so-called severe AS and low gradient.

And here is an example of, at the top of the slide, the guidelines in terms of quantifying severe AS and an example of a patient with the low valve area, tight valve area 0.4 centimeters squared, but the mean gradient here is only 30 millimeters of mercury. What to do with this kind of patient? And in the past, the outcome studies done on this patients were discordant. Here is a study by Hachicha from Canada saying that the patients with low-flow PNF is the paradoxical low flow gradient patients, they had a bad outcome.

And then in this study from Europe, the curve in blue and in orange are representing the so-called low gradients of AS, and the orange is the moderate AS. And both of these groups have the same relatively benign outcome. So we have discordant data on this patient, so it is very difficult to know what to do with a patient with low gradient. And indeed, the presentation of aortic stenosis in the elderly is challenging, not only for the low gradient, but also in patients who present with symptoms because they have co-morbidity, who present without symptoms because they don't move that much. And indeed, we need an independent method of assessment of AS severity.

And what I would like to discuss with you today is the use of aortic valve calcification. On this slide you can see a valve of aortic stenosis with the radiography of the valves showing that what is causing the aortic stenosis is the healthy deposition of calcium in the valve. And you can see by echo that we can see the calcification, but these calcification are difficult to quantify. And conversely, when we look with CT, the computed tomography allow us to see with the power of x-ray the calcification and to quantify those calcification in those patients, so that you can have a score of calcification on the aortic valve.

Here on this slide, we show a correlation between the valve area and the score of calcium that is curvilinear, showing that these patients may have-- that these two measures may have concordant use in the patient because they are not linearly related. So we see those calcification, but after all, the amount in big valves in men and in smaller valves in women should be different. Are these different in men and women in relationship to the hemodynamics? And in that study that was published in *Circulation-- Cardiovascular Imaging,* we show that, indeed, men and women are different. For the same score, the gradients are higher in women than in men. The women have the black dots. The men have the open triangles. You can see that the gradient is higher in women.

And when we look at the determinants of high AVC load, you can see that it is first the LVOT diameter, that is the size of the left ventricular outflow tract. And indeed, we have to take into account the size of the left ventricular outflow tract. But even adjusting for that, women have a different set of gradient than men. So that when we look at aortic valve density, which is the score of calcium divided by the left ventricular outflow tract area, here in men and women, we see that women reach the severe grade of aortic stenosis for lower density than men. And this has two implication-- one for research, and we'll have to understand why women have more severe your expenses for a smaller deposition of calcium, but also for the diagnosis of severe calcified aortic valve disease.

So in looking at the valve by CT, we need to take into account, absolutely, the left ventricular outflow tract size and the sex of the patient. But does that calcification quantification help in resolving the conundrum of low gradient severe aortic stenosis? Well, in that study that was published this year in the *Journal of the American College of Cardiology,* as you can see, we had a large number of patients. And most patients had concordant gradient and valve area in blue. And these patients, who had a normal ejection fraction, were analyzed for the best threshold separating the severe from the mild aortic stenosis.

And then we used that threshold to analyze the patient with discordant gradient, having either too high a gradient-- the smallest group, 2%-- or too low a gradient-- the largest group, 27% of patients. And here is the result of the study showing that the people who present with a discordant gradient and valve area, particularly with the low gradient, are not in the same category of classification as the light blue moderate aortic stenosis. They have more calcification than the moderate, yes, but less than the severe, as they are in between. And if we use the threshold defining concordant patients, we can define that approximately half of these patients, there is a severely calcified aortic valve disease that would need to be operated.

So the threshold to be defined are different in men and women. And in women, it's 300 units per centimeter squared of left ventricular outflow tract, in men, around 500 centimeter square, by centimeter square of left ventricular outflow tract. And I can tell you preliminary data that these thresholds are also predicting survival in patients with aortic stenosis. So what we have learned today is that, clinically, we have to ensure that aortic stenosis is severe, either because the valve area and the gradient are concordant to tell us that the valve is severe, or because when the area is low but the gradient does not allow the diagnosis because we find high valve calcification load, which allows us to have a diagnosis in this patient, to refer them to surgery or to percutaneous aortic valve replacement.

Now that we've seen the clinical usefulness of this patient, we understand that the measurement of aortic valve calcification is fundamental in determining aortic stenosis severity and outcome. And that, by the way, the method to measure this calcification is essential to assess not only the patient from the diagnosis point, but also for the progression of aortic stenosis. And more studies will be coming on that subject. And now we'll leave the podium to my colleague and friend, Dr. Jordan Miller, who will discuss the issue of prevention of progression of aortic valve calcification. Thank you.

DR. JORDAN MILLER: Hello. I'd like to thank Dr. Sarano for an excellent introduction into why calcification is very important clinically. And now we're going to switch gears a little bit and start to evaluate mechanisms that contribute to aortic valve calcification, and how we might be able to target those therapeutically to actually slow progression of calcification and improve the lives of patients with calcific aortic valve disease. So as the title of my talk here is, we've identified some novel mechanisms and emerging therapeutic targets in calcific aortic valve stenosis.

And so, as Dr. Sarano outlined in calcific aortic valve disease, the major problem is calcification. And so the major issue that we face as basic scientists is it's really identify mechanisms that we can target therapeutically to slow progression of calcification, fibrosis, and ultimately slow the functional progression of aortic valve stenosis. And so we know that there are several major risk factors that contribute to aortic valve disease. And these include hypertension, diabetes, hyperlipidemia, smoking, and aging. And in my research program in particular, we focus primarily on hyperlipidemia. Now, the current thinking is that, with any of these stressors, that there's an increased injury in the valve and the thelium that results in the elaboration of factors such as bone morphogenetic proteins that contribute to valve calcification and fibrosis, and ultimately aortic valve stenosis. So really it's this injury event that's key.

Now the approach that we've taken to try and understand mechanisms that contribute to valve calcification has been in animal model, and in what we use, essentially, is a more complex hypercholesterolemic mouse. And this animal, it's a low-density lipoprotein receptor-deficient, apolipoprotein B100-only mouse. So it harbors both of these mutations. And the important thing is that these animals have high cholesterol levels, and when they harbor both of these mutations there's a progressive time-dependent increase in the peak transvalvular velocity across the aortic valve, which corresponds very similarly to what is observed in humans with aortic valve disease.

Now importantly, these mice also have increases in a signaling cascade called Bone Morphogenetic Protein signaling, or BMP signaling. And in human valves, we know that this cascade is activated, and when it's activated it drives the expression of pro-calcific genes. And when we examine the valves from our mice and compare them to human valves, importantly, expression of osteogenic genes, or bone-forming genes such as Runx2, are very similar in patients with end-stage aortic valve disease, as shown on the left, and in animals with severe aortic valve stenosis, as shown on the right subset of the panels here. Now when we look at histologically and pathologically what this ends up in, is it actually-- these mice develop severe and robust aortic valve calcification similar to what's observed with humans.

So to give you just a very brief interim summary here, what we have now is we have a robust platform that allows us to investigate biological phenomena as well as use it as a model and a platform for drug testing and screening, to ultimately determine whether compounds might be useful for testing in humans to slow progression of disease. So what I hope to convince you today are really of two key things. So one is that reduced nitric oxide signaling is an important factor contributing to valve calcification in both animals and in humans. And second, that we can exploit changes in the oxidative stress and nitric oxide signaling to actually slow progression of valve calcification and stenosis, hopefully in the longer term in humans.

So our first key question here is if we alter nitric oxide signaling can we actually alter the progression of calcific aortic valve stenosis, specifically in our animal models? And so when we look at nitric oxide signaling and calcific aortic valve stenosis, we know that it's a very complex process. And really, at the center of the whole thing here is nitric oxide. But if we take a more simplified view of this, we know that nitric oxide synthases generate nitric oxide, and nitric oxide collectively acts on a variety of tissues to protect against cardiovascular disease.

Now we know that there's a molecule called asymmetric dimethylarginine that inhibits nitric oxide synthases. And importantly, this molecule is also increased in patients with calcific aortic valve disease. ADMA is decreased by an enzyme called DDAh1, and importantly, what this does is it allows us a way to alter the enzymatic activity of DDAH1, subsequently altering ADMA levels. And changing downstream levels of nitric oxide, we can evaluate experimentally what the effects of manipulating nitric oxide signaling are on the progression of cardiovascular disease. And the way that we do this is by using a perhaps even more complex hypercholesterolemic mouse.

And so what we did is we took our Ldlr-deficient, apoB100-only mice, and we crossed them with mice that either harbored a transgene of DDAH1 so it overexpressed DDAH1, which we would anticipate would be protective, or there was genetic inactivation of one copy of DDAH1. So there we would expect ADMA levels to increase, much like they are in patients with aortic valve disease, and accelerate calcification in our mice. And so these mice were fed a Western diet for six months, and at which point we measured histological changes in the valve. And we measured some functional changes in the valve using high resolution echocardiography.

And so in brief, what we showed is that when we alter the levels of DDAH1, we know that reducing levels of DDAH1 now, as shown in this figure, actually accelerates valve calcification. And that's shown with two representative micrographs on your left, and quantitated on the right there. So we get about a doubling of valve calcification.

Now when we look at functionally what this corresponds to, we can see that over time in a wild-type mouse, in the animals that have two copies of DDAH1, we can see that there is a small decrease in value function, an impairment value function over time, as measured by cusp separation distance, or how far of the cusps opened during systole. And when the animals lose one copy of DDAH1, we can see that it dramatically accelerates the rate at which valve dysfunction progresses. Now when we overexpressed DDAH1, you can see here that at early time points there might be some moderate protection against valvular dysfunction, but as we get out to the six month time point, again, we really don't see a significant impact compared to the wild-type animals.

So we view this as being both good and bad news. So when we reduce nitric oxide signaling, the good news is that we accelerate valve disease and it provides a very solid proof of concept. The bad news is that when we increase nitric oxide signaling by overexpressing this molecule that we thought would be protective, we did not observe consistent or robust improvements in valve function. And our question then was, well, why not? And so the answer is, well, maybe it's not as simple as we thought it initially was.

And if we focus in maybe a little bit more closely on this downstream signaling cascade related to nitric oxide signaling, we know that nitric oxide synthase requires certain co-factors like BH4, or tetrahydrobiopterin. And nitric oxide subsequently acts on an effector molecule called soluble Guanylate Cyclase, or sGC. Now when there are increases in oxidative stress, which are a near ubiquitous finding that patients with calcific aortic valve stenosis, this can not only reduce tetrahydrobiopterin levels, but also reduce nitric oxide bioavailability and also oxidize sGC.

So we really have three areas that we have to overcome before we can even start to increase the effectiveness of nitric oxide signaling. And so what we thought was that we should actually focus in on the lowest common denominator, and perhaps focus on sGC to circumvent all the problems associated with the bioavailability of cofactors for nitric oxide synthase. So the thought is that when sGC is in its native form and there are low levels of oxidative stress, that nitric oxide can bind to sGC and protect against cardiovascular disease. Now, when oxidative stress is increased, sGC is oxidized and it becomes very nitric oxide insensitive.

Now interestingly, there's a new class of molecules that may allow us to actually circumvent this entire system. And one example of this would be an anthranilic acid derivative called Ataciguat, which activates soluble guanylate cyclase when it's in its oxidized form, and thereby circumvents this whole system, and should protect against cardiovascular disease and aortic valve calcification in particular.

And so what we currently have is a UH2/UH3 grant from NIH that focuses on the use of Ataciguat to slow progression of calcific aortic valve stenosis. And this is a collaboration with myself, Dr. Sarano, and Dr. Hartzell Schaff in the department of surgery. So in our first year what we aim to do is generate preclinical work showing proof of concept as well as tolerance testing in humans. And then years two and three, what we aim to do is start a clinical trial in humans with calcific aortic valve disease to examine the efficacy of this drug on slowing progression of valve calcification in humans.

So when we look at some of our preliminary findings in vitro, what we found is that with bone morphogenetic protein signaling, we know that this signaling cascade is activated in valves from patients with aortic valve disease, and that it drives the expression of a known osteogenic molecule called SP7. When we look at the panel at the right, what you can see is that when we treat with BMP-2-- and the lower panel, you can see that we have upregulation of this molecule called SP7. Now in the blot in the middle, there's a molecule called phospho-VASP 239. Now, phospho-VASP 239 is upregulated when sGC signaling is activated.

So you can see that bone morphogenetic protein signaling per se doesn't suppress nitric oxide signaling or sGC signaling in the system. Now when we treat with Ataciguat, we can see that we get robust activation of phospho-VASP 239, which suggests that sGC signaling is very active. And importantly, when we measure SP7 levels in these cells in vitro, we can then see that SP7 expression is virtually abrogated, and the reduction of SP7 by BMP-2 is virtually nonexistent.

So what about in vivo? Well, we have our animals currently, these Ldlr-deficient, apoB100-only mice. They're placed on the Western diet. We've placed them on that for six months, where we're either treating with no drug or Ataciguat, and these studies are ongoing. And we plan on reporting these data in the very near future.

So where are we going from here? Well there's extensive safety testing that has already been conducted in humans with other medical conditions and cardiovascular diseases. And this is a result of previous preclinical testing and clinical testing conducted by Sanofi pharmaceuticals, who are the makers of this compound. So we're in the process of conducting a small pilot study at Mayo Clinic in Rochester, Minnesota. And importantly, this trial and this pilot study will perform two key purposes. One is to develop potential biomarkers of drug activity so we can monitor the activity of patients over time in a longer-term trial. And two is to confirm the safety of the compound in patients with calcific aortic valve stenosis, where we anticipate this compound will be very safe.

So currently, this tolerance testing is starting. Our inclusion criteria include patients with an aortic valve area between 1 and 2 centimeters squared, and an ejection fraction greater than 50%, and an age greater than 50. And essentially, our protocol involves orthostatic tolerance testing, where patients simply sit in a semi-recumbent position and stand up a handful of times. They undergo mild tilt table testing to assess blood pressure responses to gentle tilting. And we do this before and after 14 days of either Ataciguat or placebo treatment. And we are currently recruiting for these studies.

So to summarize, nitric oxide signaling is mechanistically important in the pathogenesis of calcific aortic valve stenosis and plays an active role in the suppression of known signaling cascades that promote calcification in the valve. Furthermore, we believe that nitric oxide signaling may be harnessed therapeutically to slow progression of valve disease in patients with calcific aortic valve stenosis, using this newer class of drugs known as anthranilic acid derivatives.

Thank you very much for your time today. And I hope that this talk has been helpful in helping you understand some of the research that is going on at Mayo Clinic to improve the lives of patients with calcific aortic valve disease.