

Experimental aortic valve stenosis in rabbits

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Abstract :

Background : Aortic valve stenosis (AS) is the most common valvular heart disease. Recent observations have suggested a link between atherosclerosis and the development of AS. However, until now, there has been no solid direct proof of this potential link.

Objectives: We studied a known animal model of atherosclerosis and hypercholesterolemia, the cholesterol-fed rabbit, to assess the effect of this diet on aortic valve morphology and function.

Methods: Rabbits were divided in three groups: 1) no treatment; 2) cholesterol-enriched diet (0.5% cholesterol) and 3) cholesterol-enriched diet + vitamin D₂ (50000IU daily). Echocardiographic assessment of the aortic valve was done at baseline and after 12 weeks of treatment. Aortic valve area, maximal and mean transvalvular gradients were recorded and compared over time.

Results: Control animals displayed no abnormalities of their aortic valve. Despite important increases in blood total cholesterol levels, animals in group 2 did not develop any significant functional aortic valve abnormality over 12 weeks. However, 8/10 of the animals in group 3 developed a significant decrease in aortic valve area ($p=0.004$) and significant increases in transvalvular gradients ($p=0.003$).

Conclusion: This study supports a potential link between atherosclerosis and the development of AS. The differences noted between hypercholesterolemic animals with or without vitamin D₂ implies a significant role of calcium in the development of AS that deserves further attention.

Key words:

Aortic valve stenosis, hypercholesterolemia, rabbits, sclerosis, calcification, echocardiography, vitamin D.

Condensed abstract:

A link between atherosclerosis and the development of aortic valve stenosis (AS) has been suggested. We studied cholesterol-fed rabbits receiving or not vitamin D₂ and assessed their aortic valve (AV) function. Echocardiographic assessment of the aortic valve was done during treatment. Control and cholesterol-fed animals developed no abnormality of their valve. However, animals receiving cholesterol and vitamin D₂ developed a significant decrease in AV area and significant increase in transvalvular gradient. This study supports a potential link between atherosclerosis and the development of AS. The differences noted between hypercholesterolemia with or without vitamin D₂ imply a role for calcium.

Introduction:

Aortic valve stenosis (AS) is the most common valvular heart disease in Western countries. Considering its increasing prevalence with age, aortic valve stenosis is bound to become an ever increasing health problem in aging populations. Since AS was always considered to be simply a degenerative disease of the valve, virtually no attention was given to its potential medical treatment other than surgical replacement of the valve in severe symptomatic cases. However, many recent observations have suggested a link between the pathophysiology of the development of AS and atherosclerotic disease: in cohort studies, many traditional atherosclerotic risk factors have been associated with increasing prevalence of aortic stenosis (1-6). Therefore, many investigators now center their efforts on the hypothesis of an atherosclerosis-AS link.

Animal models of AS have been developed in the past but all of those models implied a surgical manipulation of the aortic valve *per se* or banding of the ascending aorta. None of these invasive animal models reproduce the true pathophysiology of native AS. Moreover, none of these models allow the study of the evolution of neither the early phases of aortic valve disease nor the potential prevention of progression to severe stages of the disease. In this study, we report for the first time the development of AS in a rabbit model of atherosclerosis.

Methods:

Animals:

22 male New Zealand rabbits (2-2.5 kg) were divided in three study groups (six to 10 animals / group): 1) controls receiving normal rabbit chow without any dietary supplement; 2) animals fed with 0.5% cholesterol (chol)-enriched chow (Harlan, Indianapolis, IN) and 3) animals fed with 0.5% cholesterol-enriched chow plus 50000 IU vitamin D₂ (Sigma, Markham, Ont. Canada) daily in drinking water. Vitamin D₂ has been shown in other models to accelerate the atherosclerotic process. All animals were treated in accordance with the recommendations of the Canadian Council for Animal Care. The protocol was approved by the Université Laval Animal Protection Committee. Blood samples were taken via the marginal vein of the ear at baseline and weekly thereafter for the measurement of cholesterol as well as calcium levels in serum.

Echocardiography, aortic valve area and transvalvular gradient measurements:

Echocardiography was performed at baseline and 12 weeks after the beginning of treatment. To perform the echocardiogram, animals were sedated with an intramuscular injection of ketamine (30 mg/kg), midazolam (0.5 mg/kg) and butorphanol (0.5 mg/kg). The chest was shaved and the animals put in a dorsal decubitus position. Ultrasound images were obtained with a 12MHz phased-array probe connected to a Sonos 5500 echograph (Philips Medical Imaging, Andover, MA).

Aortic valve area was measured by the standard continuity equation as currently recommended in the literature in the same manner that is performed in humans with suspected aortic valve stenosis. Parasternal long axis view was used to measure the

diameter of the left ventricular outflow tract as well as to observe the morphology and opening of the aortic valve. The aortic valve was also imaged in the short axis view to assess leaflet morphology. An apical five chamber view was obtained in order to measure the stroke volume with pulsed-Doppler proximal to the aortic valve as is routinely done in humans. Continuous-wave Doppler was used to record maximal and mean aortic transvalvular gradients as well as to obtain the integral of transvalvular flow to be used in the continuity equation for aortic valve area calculation. All echocardiographic imaging and analysis was performed throughout the protocol by the same investigator experienced in performing echocardiographic studies in rabbits and other small animals. Inter and intra-observer variability was assessed blindly on half of the animal studies for aortic valve area measurement.

Histology: At the end of protocol, rabbits were exsanguinated under anesthesia. The ascending aorta section containing the aortic valve was dissected, rinsed in phosphate-buffered saline, embedded in OCT and snap-frozen in liquid nitrogen. Cross-sectional serial sections were prepared and Alizarin red S and von Kossa silver stains were carried out to visualize calcium deposition.

Statistics:

Results are presented as mean \pm SEM unless specified otherwise. Repeated measure one-way ANOVA with post hoc Tukey's test or paired *t* tests were used for intragroup comparisons to assess the evolution of Ca²⁺ serum levels over time. Unpaired *t* test was used for intergroup comparisons (Total cholesterol levels). Paired *t* test was used for

comparisons of the effect of diet on echocardiographic parameters. Statistical significance was set at $P < 0.05$.

Results:

Cholesterol and calcium levels:

Figure 1 depicts the evolution of total cholesterol as well as calcium circulatory levels in the three animal groups. Animals from group 1 displayed no modifications of those two parameters. Total cholesterol circulatory levels rose to high levels in both groups 2 and 3 (Fig 1a). Interestingly, vitamin D₂ caused an additional increase of serum cholesterol despite similar cholesterol intake. Calcium levels were slightly more elevated in the animals from group 3. This mild difference reached statistical significance from week 8 although calcium levels were elevated throughout the protocol (Fig 1b).

Development of atherosclerosis:

Arteries of the control group remained normal throughout the protocol. As expected based on previous observations by other groups who used this model, the animals fed with the cholesterol-enriched diets (with or without vitamin D supplements) developed severe atherosclerotic involvement of their arteries that was evident at macroscopic as well as microscopic examination (not shown).

Aortic valve:

Results for aortic valve area are summarized in Figure 2. Baseline aortic valve areas were comparable in all three groups. When corrected for body weight gain, no significant change was noted in calculated valve area in animals in groups control and chol throughout the protocol (Fig 2, middle column). However, animals in group chol + vit D₂ behaved differently (Fig 2, bottom row). Aortic valve area (AVA) decreased in eight out of ten animals after 12 weeks of treatment. This decrease was even more pronounced when AVAs were corrected for the animal's weight. Transvalvular gradients

remained normal in groups control and chol but increased in the chol + vit D₂ group significantly during the protocol as aortic valve area decreased. An example is illustrated in Figure 3. Two-dimensional imaging of the diseased aortic valves showed an increase in the thickness and echogenicity of the leaflets as well as a reduced mobility compatible with leaflet sclerosis and areas of calcification. This is confirmed by visual inspection of excised valves as illustrated in Figure 4. Histological examination of the diseased valves demonstrated leaflet thickening and deposition of calcium both at the aortic attachment site as well as in the leaflet body. An example of aortic valve echographic abnormalities seen in animals from group 3 compared to control animals is given in Figure 5. Histological evidence of calcium deposition is displayed in Figure 6. Inter and intra-observer variability was blindly assessed on half of the animals for the echocardiographic measurement of aortic valve area by continuity equation. Intra-observer variability was less than 5% and inter-observer variability was 8%.

Discussion:

The theory of an atherosclerotic pathogenesis of AS in humans is becoming more and more popular. AS had been described many years ago in patients suffering from cholesterol metabolism abnormalities such as Tangier's disease and severe homozygous familial hypercholesterolemia (7-9). Following this lead, several recent publications have demonstrated a clear link between atherosclerosis *per se* as well as specific cardiovascular risk factors such as hypercholesterolemia, systemic hypertension and diabetes mellitus (1;2;4;6;10-14). Unfortunately, most of the evidence currently available relies on observational cohort studies. Prospective studies are clearly needed to assess the effect of those risk factors as well as the potential effect of pharmacological treatment on the evolution of AS.

In this study, we have been able to induce peripheral atherosclerosis and, more importantly, an aortic valve stenosis in animals fed with a cholesterol-rich diet supplemented with vitamin D₂. Aortic valve leaflet thickening as well as reduced systolic motion of those leaflets was clear in two-dimensional echographic imaging, transvalvular gradients increased significantly compared to controls and, most importantly, aortic valve opening was significantly reduced at echographic two-dimensional imaging and this was confirmed by the decreased valve area calculated by continuity equation in those animals.

To our knowledge, this is the first description of a clear prospective link between atherosclerosis and AS in an animal model. Previous studies had demonstrated an atherosclerotic involvement of the aortic valve in hypercholesterolemic animal models(15-23). Unfortunately, since aortic valve pathology was not the scope of those

publications, the *in vivo* evaluation to detect a potential dysfunction of the valve was never investigated in those animals.

As for atherosclerotic plaques, the mechanisms of aortic valve calcification that contribute to the progressive stenosis of the valve are still incompletely understood. In this protocol, we used vitamin D supplementation in combination with a hypercholesterolemic diet, a supplementation that has been used for decades by investigators studying hypercholesterolemic rabbit models of atherosclerosis to enhance and accelerate the development of the calcification process (24-30). It has also been shown that vitamin D metabolites may induce a disruption in the integrity of the arterial wall as well as some degree of smooth muscle necrosis of the arterial media of swine as well as rabbits, thereby enhancing the integration of lipid particles by the arterial wall (31-33). This mechanism may explain the accelerating effect of vitamin D on atherosclerosis development in hypercholesterolemic rabbits. Conversely, calcium channel blockers have been proven to delay the development of atherosclerosis in hypercholesterolemic rabbits (24). We hypothesized that the aortic valve leaflets would demonstrate the same behavior when subjected to hypercholesterolemia and vitamin D supplementation and that animals treated with this combination would develop more severe aortic valve abnormalities earlier than those treated with a high cholesterol diet alone. Our results confirm this hypothesis. Indeed, animals not receiving supplements of vitamin D₂ did not display any significant degree of AS after 12 weeks of treatment despite very high levels of blood cholesterol (above 20 mmol/l) and presence of peripheral atherosclerotic lesions. In humans, vitamin D overload has been mostly associated in the past with supra-valvular AS which was not the main lesion in our animals in whom stenosis of the aortic valve itself was clear (34-38). Interestingly, a

recent observation links a vitamin D receptor gene polymorphism to calcific aortic stenosis in humans (39).

Our model has many advantages over most of those previously described. In small animals, pressure-overload models with banding of the ascending aorta as been used to mimic the situation seen in valvular stenosis (40-44). However, this model has the disadvantage of producing a supra-coronary obstruction that is not reflective of the situation of valvular stenosis where the obstruction occurs before the implantation of the coronary arteries. In larger animals such as sheep, aortic valve stenosis has also been induced by surgical procedures. Since the disease is artificially created, it is impossible to study the physiopathology the disease on the valve as well as potential strategies for preventing or slowing the degenerative process. We have developed a model where the pathogenesis of AS is probably related to the atherogenic factors that seem to be the mechanism also suspected in human disease. All animal models have their limitations and, of course, we are aware that the animals in this protocol were submitted to extreme situations of hypercholesterolemia together with vitamin D supplementation, a combination that will not be encountered in humans. Multiple risk factors that are clearly absent from our model interact in humans to induce AS. The long-term evolution of the animals has not been assessed and we still do not know if the disease will continue to progress over time. Reversibility has not been assessed nor the effect of withdrawal of the offending factors.

Conclusions:

We report an experimental model of acquired aortic valve stenosis in rabbits fed with a high cholesterol diet and supplements of vitamin D₂. These results support the hypothesis of the link between atherosclerosis and aortic valve stenosis in humans.

This experimental model may prove useful to study the early evolution of the disease, to study the effects of various atherosclerotic risk factors on this evolution or the use of pharmacological treatments to delay or even stop this evolution.

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Figure Legends:

Figure 1: Total cholesterol (A) and Ca^{2+} (B) circulatory levels in hypercholesterolemic rabbits receiving (○) or not (●) daily supplements of vitamin D_2 . Results are expressed as mean \pm SEM in mmol/l (mM). (n=8). The hatched region in graph B corresponds to the mean (line) \pm 2 SD of serum calcium concentrations in normal rabbits (n=18).

Figure 2: Effects of hypercholesterolemia in rabbits receiving (Chol+Vit D_2) or not (Chol) daily supplements of vitamin D_2 on aortic valve function as assessed by echocardiography. Aortic valve area (first column) and indexed aortic valve area (middle column) (arbitrary units (AU)) and maximal gradient (mmHg) were calculated as described in the Methods section and are represented for each individual rabbit. ns: not significant.

Figure 3: Two dimensional imaging of the aortic valve by echocardiography. The valves were imaged in the parasternal long axis (left panels) and short axis (right panels) views to assess leaflets morphology. In control animals (Control), the aortic valve is a thin structure barely visible. In animals fed with cholesterol and vitamin D supplements (Chol + Vit D_2), the aortic leaflets become thickened and hyperechogenic. Leaflet valve area are reduced as shown by the limited opening of the valve in systole. Valve opening was traced over the echocardiographic image only as a visual help to assess leaflet motion and valve opening and aortic valve area was not calculated from those tracings.

Figure 4: Aortic valve photographs from rabbits fed with normal chow (left) or cholesterol+vitamin D₂ (right).

Figure 5: Transvalvular aortic gradients in control (top panel) and cholesterol + vitamin D₂ animals (lower panel) by continuous wave Doppler imaging.

Figure 6: Calcium deposition as assessed by alizarin red S (left) and von Kossa (right) staining in the attachment site (B, F), aortic valve leaflet (C, G) as well as in the aortic annulus (D, H) in rabbits treated with cholesterol enriched diet + vitamin D₂. Sections from a normal aortic valve are shown in panels A and E.

Figure 1 Drolet et al.

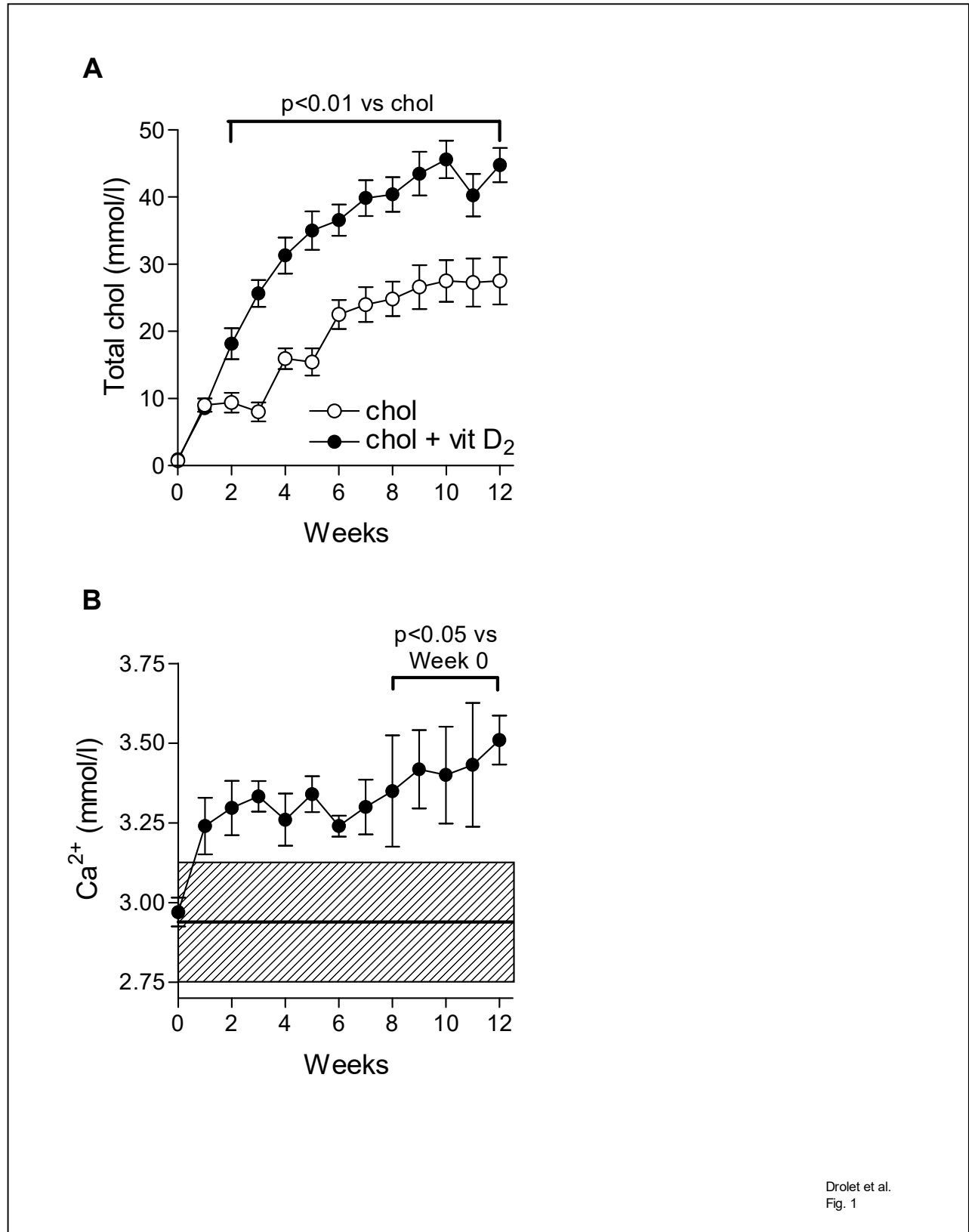
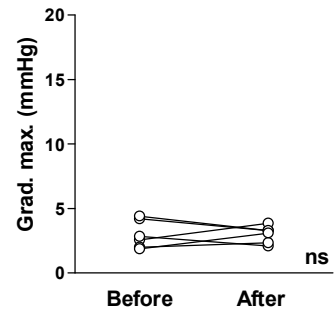
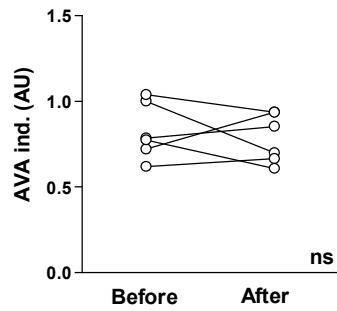
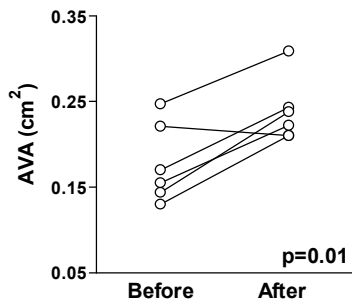
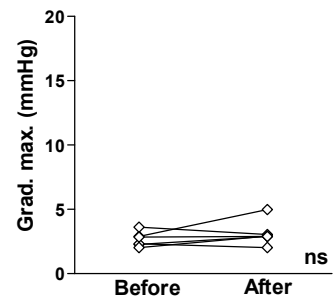
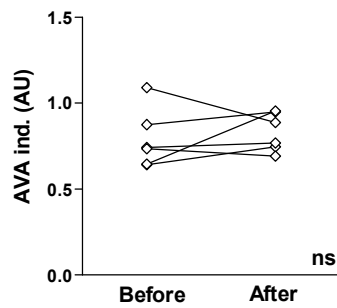
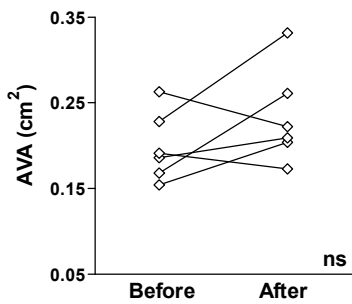


Figure 2 Drolet et al.

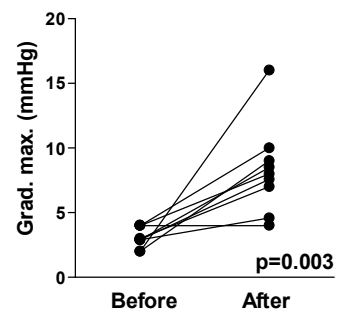
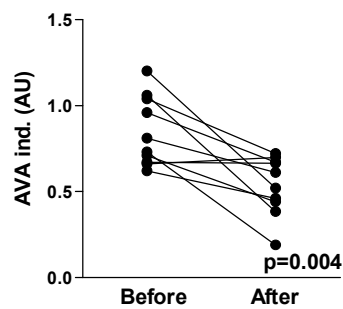
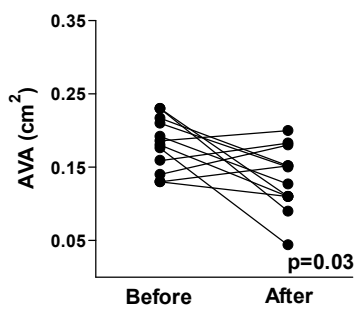
Control



Chol



Chol+vit D₂



**Aortic valve
area**

**Aortic valve
area index**

**Maximal
gradient**

Figure 3 Drolet et al.

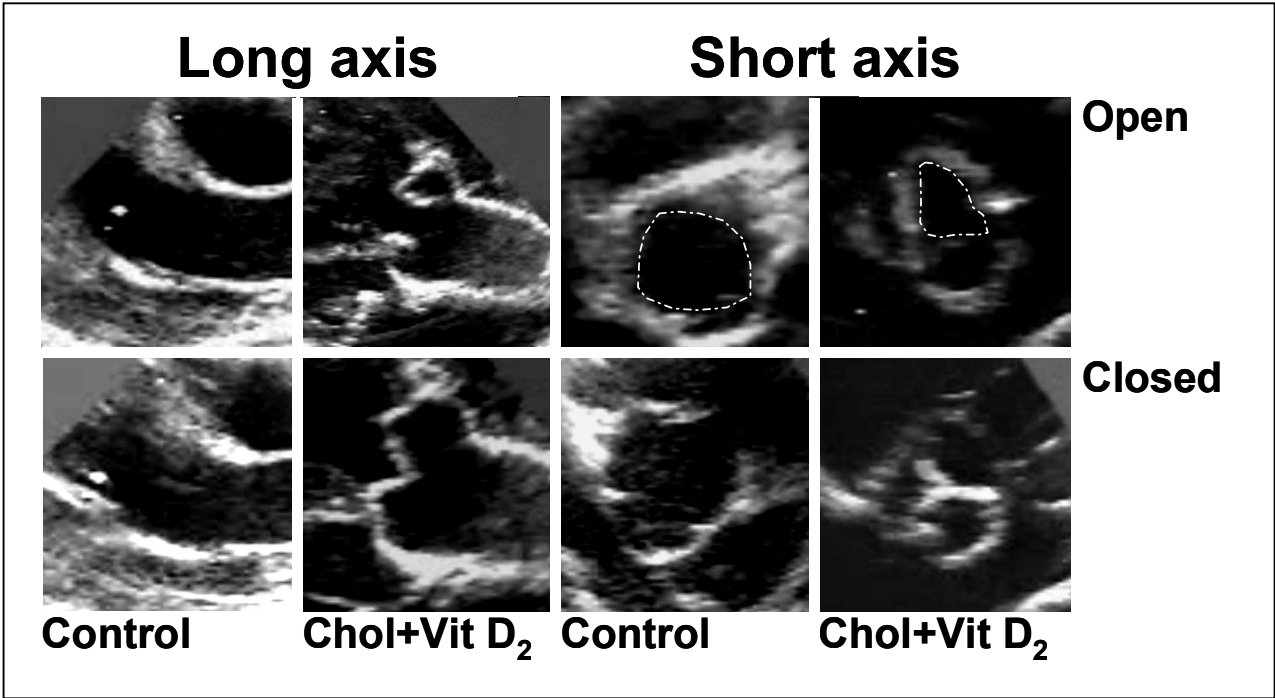


Figure 5 Drolet et al.

