A Western high fat/high carbohydrate diet induces aortic valve disease in C57BL/6J mice

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Background: Observations suggest a link between aortic valve stenosis (AS) and atherosclerosis. AS has been induced in animal models of extreme hypercholesterolemia but such high levels of blood cholesterol are infrequent in humans making those experimental models less relevant. It is not known if a pro-atherogenic high fat/high carbohydrate (hf/hc) Western diet without added cholesterol could have the same negative impacts on the valve.

Objectives: To compare aortic valve function and morphology in normocholesterolemic adult wild-type (wt) C57BL/6J mice and severely hypercholesterolemic LDL receptor-deficient (LDLr-/-)) mice fed or not with a hf/hc diet.

Methods: Animals were divided in four groups (n=10-11/group): 1: wt+normal diet; 2: wt+hf/hc; 3: LDLr-/-+normal diet; 4: LDLr-/-+hf/hc. Valve area and transvalvular velocities were evaluated by echocardiography after 4 months. Valves were then collected for tissue analysis.

Results: Wt on hf/hc diet became mildly hypercholesterolemic, obese and hyperglycemic. As expected, all LDLr-/- were severely hypercholesterolemic. Animals on hf/hc (gr.2, 4) displayed smaller valve areas and higher transvalvular velocities ($p<0.01$) compared to animals on normal diet. Aortic valve leaflets were thicker and infiltrated with lipids and macrophages in both hf/hc groups (wt and LDLr-/-).

Conclusion: Four months of a Western diet in mice results in significant aortic valve abnormalities. Severe hypercholesterolemia was not a pre-requisite in this model. Wt+hf/hc reproduces aortic valve disease with a combination of atherogenic factors more commonly encountered in humans than isolated extreme hypercholesterolemia. This experimental model suggests that AS is multi-factorial and that hypercholesterolemia should not be the only target in this disease.

Condensed abstract

Until now, experimental aortic stenosis models implied severe hypercholesterolemia by feeding animals high amounts of cholesterol. We evaluated if a Western high-fat (no added cholesterol)/high carbohydrate diet could alter the aortic valve in normocholesterolemic (wild type (WT)) or hypercholesterolemic (LDL receptor-deficient (LDLr-/-)) mice. The Western diet induced mild hypercholesterolemia, obesity and hyperglycemia in WT and severe hypercholesterolemia and diabetes in LDLr-/-. Both groups had smaller valve areas and higher transvalvular velocities than corresponding animals on normal diet. Leaflets showed significant tissue abnormalities in all animals on Western diet. In this mouse model, 4 months of mild hypercholesterolemia, obesity and hyperglycemia significantly affected the aortic valve.

Key words:

Aortic valve stenosis, echocardiography, obesity, C57BL/6J mice, sclerosis, hypercholesterolemia.

Introduction:

Degenerative aortic valve stenosis (AS) is the most common valvular heart disease in Western countries and has long been considered as purely degenerative. However, recent observations have suggested a link between AS and atherosclerosis. Many traditional atherogenic factors such as hypercholesterolemia, obesity and diabetes have been associated with a higher prevalence of aortic stenosis (1-3). Therefore, many investigators focus on the hypothesis of an atherosclerosis-AS link.

Hypercholesterolemia has been recurrently incriminated as risk factor for developing AS. Cholesterol lowering drugs (statins) are currently under investigation in a few prospective trials evaluating their potential to slow or halt AS progression (4-7).

Investigators have tried to reproduce AS in animal models of atherosclerosis in order to look more deeply into this AS-atherosclerosis link. We and others have recently reported significant aortic valve abnormalities both *in vivo* and *in vitro* in a classical experimental atherosclerosis model: the cholesterol-fed New-Zealand rabbit (8-11). This experimental animal model of extreme hypercholesterolemia leads to characteristic atherosclerosis-like lesions in the aortic valve leaflets and, when combined with a pro-calcifying agent, to a progressive reduction of aortic valve area and increase in transvalvular velocities compatible with the early stages of aortic valve stenosis. This type of animal model remains very useful but, unfortunately, it does not closely reproduce the human disease since such high levels of blood cholesterol are rarely if ever encountered in humans.

Considering that AS has been statistically associated with milder metabolic abnormalities in human retrospective studies, we hypothesized that a less aggressive atherogenic diet, without high cholesterol contents, may significantly alter the aortic valve. The current

study was therefore designed to assess if a Western high fat-high carbohydrate (hf/hc) diet without added cholesterol can alter the aortic valve's function and morphology in mice. C57Bl/6J mice which are genetically prone to develop diet-induced obesity and atherosclerotic lesions were chosen for this protocol. This diet was tested on both normo-cholesterolemic (wild type or wt) and hypercholesterolemic C57Bl/6J mice deficient in LDL receptors (12) (LDLr -/-) to see if severe hypercholesterolemia was a pre-requisite to induce aortic valve disease in those animals.

Methods:

Animals:

42 adult male C57BL/6J mice (20-25 g), wild type (wt) or LDL receptor deficient (LDLr-/- in the C57BL/6J background) (Jackson Laboratories, Bar Harbor, MA), were divided in four groups (10-11 animals/group): group 1: wt + normal diet; group 2: wt + high fat/high carbohydrate diet (hf/hc); group 3: LDLr-/- with normal diet and group 4: LDLr-/- + hf/hc diet. All animals were followed for 4 months. This protocol was made in accordance with the recommendations of the Canadian Council for Animal Care and was approved by the Université Laval Animal Protection Committee.

Diets:

Normal diet consisted of normal mouse chow (Mouse Colony Chow #5018, Purina Mills, MO, USA). The High fat/High carbohydrate (hf/hc) diet was purchased from BioServ #F3282, (Frenchtown NJ, USA). In this hf/hc diet 59% of total calories are provided from fats (high palmitic acid contents) without added cholesterol. Both normal and hf/hc diets were available to the animals without restriction.

At the end of protocol, mice were fasted overnight and exsanguinated under ketamine/xylazine anesthesia. Blood samples were taken for the measurement of fasting total cholesterol, glucose as well as insulin levels. Plasma levels of TNFα and interleukin-6 (IL-6) were also evaluated using specific mouse enzymatic immunoassays (Assay Designs, Inc., Ann Arbor, MI).

Echocardiography:

Echocardiography was performed at baseline and 16 weeks after the beginning of the protocol under ketamine/xylazine anesthesia. 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 continuity equation as previously published. 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. All echocardiographic imaging and analysis were performed throughout the protocol by the same investigator experienced in performing echocardiographic studies in such small animals.

Histology:

At sacrifice, a portion of the ascending aorta including the aortic valve was dissected, rinsed in phosphate-buffered saline, fixed in paraformaldehyde and mounted in paraffin. Cross-sectional serial sections were prepared and von Kossa silver stain was carried out to visualize calcium deposition.

Aortic valve leaflet thickness:

Good quality leaflet sections of each group (n=5/group) were selected and leaflet thickness (middle portion, away from insertion point with aorta) was measured using an image analysis software (SigmaScan, Systat Software, Inc. Point Richmond, CA) by an investigator blinded to the treatment group. Normal leaflets (wt mice under normal diet (group 1)) were used as controls and the leaflet thickness of this group was arbitrarily set

at 100%. Leaflet thicknesses of the other groups were expressed in % relatively to group 1.

Immunohistochemistry:

The avidin-biotin-horseradish peroxidase system (Vectastain, Vector Laboratories, Burlingame, CA) was used for immunostaining. Monoclonal mouse anti-CD68 antibodies (NeoMarkers, Fremont, CA) were used followed by a secondary peroxidase conjugated rabbit anti-mouse antibody (Vectastain) for the identification of macrophage cells. Labeling for osteopontin (Clone 1B20, Assay Designs Inc., Ann Arbor, MI) and T-lymphocytes (anti-CD3, clone UCHT1, DAKO) were performed similarly.

Statistics:

Results are presented as mean ± SEM unless specified otherwise. Repeated measure one-way ANOVA with post hoc Tukey's test was used for inter-group comparisons over time. Unpaired *t* test was used for comparisons between two groups. Statistical significance was set at $p<0.05$.

Results:

Animal characteristics: (Table 1)

Both diets were well tolerated and all animals survived the 16 weeks.

LDLr-/- mice on normal diet (group 3) did not gain any weight during the protocol and therefore remained significantly smaller than all the other groups. Their heart, lung, and liver weight behaved accordingly and remained smaller than those of the other groups. All animals on the hf/hc diet in groups 2 and 4 (wt and LDLr-/-) were significantly overweight compared to controls in group 1 (wt+normal diet). At sacrifice, total heart weight was significantly increased in both the wt and LDLr-/- on hf/hc diet as well as their liver weight.

Fasting cholesterol, glucose and insulin levels (Table 2):

Cholesterol: Compared to controls in group 1 (wt + normal diet), cholesterol levels were mildly elevated in group 2 (wt + hf/hc diet) to levels comparable to group 3 (LDLr-/- + normal diet, p=ns group 2 vs. 3) and extremely elevated in group 4 (LDLr-/- + hf/hc diet, $p<0.01$ vs. all other groups). LDL cholesterol levels were increased in the wt+hf/hc diet (group 2) compared to group 1 but these levels remained relatively low. As expected, LDL levels in groups 3 and 4 were higher than group 1 and 2, the highest levels found in group 4.

Glucose and insulin levels: Fasting glucose and insulin levels remained similar to controls (group 1) in the wt animals fed with the hf/hc diet (group 2). Insulin tended to be slightly lower in group 2 but this did not reach statistical significance. Fasting blood glucose was higher than normal reported values for fasting glucose in normal mice in both groups 1 and 2. Insulin levels were however within normal reported values for mice in those two groups. The resulting glucose/insulin ratio was suggestive of insulin resistance (high glucose with normal insulin levels). Both LDLr-/- groups (groups 3 and 4) had very high blood glucose levels combined with severely decreased insulin levels compatible with overt diabetes mellitus with decreased insulin production.

Mediators of inflammation:

Inflammatory mediators IL-6 and TNFα were measured and remained at low levels, without any significant difference between group 1 and 2.

Aortic valve (Table 3 and Figures 1-3):

In vivo echocardiographic assessment:

Echocardiographic measurements are summarized in table 3. Aortic valve leaflets were more echogenic after 16 weeks in groups 2, 3 and 4 compared to group 1. Aortic valve area was significantly smaller in all groups (2, 3 and 4) when compared to controls (group 1, wt + normal diet). Considering the large differences in body weight between groups, valve area was indexed for body weight as shown in Table 3. Once corrected for body weight, valve area in group 3 (LDLr-/- with normal diet) was not reduced and remained comparable to group 1. Only animals on hf/hc diet, wt and LDLr-/-, had smaller valve

areas despite correction for body weight. Similar results were found for maximal transvalvular velocities (Table 3). Only animals on the hf/hc diet (groups 2 and 4) displayed increased transvalvular velocities whereas animals on normal diet (group 1 and 3) remained with normal transvalvular velocities. Figure 1 A and B shows the parallel evolution of aortic valve area and body weight over time during the 4 month protocol in groups 1 and 2. In group 1, both body weight and valve area increased during the course of the protocol while group 2 animals gained more weight but their valve area remained unchanged. Increases in transvalvular velocities became significant between 2-4 months of the hf/hc diet (Figure 1C). Typical examples of transvalvular velocities in group 1 (top) and 2 (bottom) are shown in figure 2.

Leaflet thickness (Figures 3 and 4):

Leaflet thickness was significantly increased in groups 2 and 4 (hf/hc diet) compared to group 1 (wt on normal diet). There was no significant leaflet thickening in group 3. A typical example of leaflet thickening in group 2 is illustrated in Figure 4.

Immunohistochemistry (Figure 4)

Macrophage infiltration (CD-68 positive cells) and foam cells were clearly evident in the thickened valve leaflets of the animals in group 2 and 4. Osteopontin expression and T-lymphocyte infiltrates were also detected as well as some nodular calcium deposition by Von Kossa staining.

Discussion:

We report for the first time in an animal model that aortic valve disease can be initiated by a Western high fat/high carbohydrate diet. Contrarily to previously published animal models of the disease who were exclusively associated with severe hypercholesterolemia, we demonstrate in this study not only that a hf/hc diet with a low cholesterol content results in mild dyslipidemia, obesity and hyperglycemia in these mice but also induces significant aortic valve abnormalities both *in vivo* (smaller valve area and higher transvalvular velocities) and *in vitro* (leaflet thickening, lipid and macrophage infiltrates, signs of calcification). This thickening of the leaflets combined with calcification probably results in an increase in the inertia of the leaflets, increase in leaflet rigidity and, therefore, increased transvalvular velocities and reduced opening of the valve. The abnormalities found in the valves of the animals closely resemble those found in the early phases of aortic valve stenosis (13;14).

For many years, aortic stenosis was considered a “wear and tear” phenomenon without any potential for medical treatment other than valve replacement surgery when symptoms or left ventricular dysfunction occurred. However, numerous epidemiologic and histological studies published in recent years have pointed to a close link between pro-atherogenic factors and the prevalence/progression of aortic valve stenosis (1;15;16). Dyslipidemia (high LDL, low HDL, high lipoprotein a), cigarette smoking, obesity, diabetes mellitus, hypertension and inflammation (high CRP levels) have all been shown to be statistically associated with aortic valve stenosis(3;5;6;17-22).

Hypercholesterolemia has been recurrently associated with aortic stenosis both in epidemiologic studies and histological studies who clearly demonstrated LDL deposits in

the diseased leaflets (23). Cholesterol-lowering statin therapy as a mean of slowing aortic stenosis progression is currently under investigation in a few prospective trials worldwide. In order to better understand the pathophysiology of the disease and its link with hypercholesterolemia, animal models of aortic valve sclerosis/stenosis have recently been developed (9-11). It has clearly been shown in these animal models of extreme hypercholesterolemia that aortic valve leaflet lesions similar to those found in humans can be reproduced (9-11). However the development of aortic valve stenosis in severely hypercholesterolemic animals is not reminiscent of the human disease since such high levels of blood cholesterol are rarely if ever encountered in humans. These animal models remain very useful but are clearly imperfect.

Numerous unanswered questions regarding the true role of hypercholesterolemia in AS remain unanswered. For example, patients with familial hypercholesterolemia mostly develop a supra-valvular type of stenosis and not the classical aortic valve disease. The treatment of hypercholesterolemia to halt the progression of aortic valve stenosis remains controversial despite a clear statistical association in retrospective and epidemiologic studies. One recent published prospective trial with a statin has yielded deceiving results (24). Aortic valve stenosis is clearly a frequent disease in the Western world and the pro-atherogenic factors that have been statistically linked with AS are closely related to the Western lifestyle. Hypercholesterolemia is only one of these factors.

For these reasons, we sought to determine if the exposition to a classical Western diet made of high amounts of fats and carbohydrates could induce significant abnormalities in the aortic valve of mice. This type of diet induces obesity, mild hypercholesterolemia and hyperglycemia in the animals which is more reminiscent of the classical atherogenic factors found in Western countries. This combination of obesity, hypercholesterolemia

and glucose intolerance/diabetes resembles what is called in humans called the metabolic syndrome (25)

Considering that previous animal models of the disease were related to severe hypercholesterolemia also wanted to assess if this was a pre-requisite for the development of aortic valve lesions. We therefore compared normal mice with mutants deficient in the LDL receptor. A high fat/high carbohydrate diet had been reported to induce vascular calcified atherosclerotic lesions in those mutant mice. Our results show that aortic valve abnormalities are found in both groups (wild type and LDLr-/-, groups 2 and 4) on the hf/hc diet despite very different LDL cholesterol levels and LDL nearly 60 times. LDLr-/- animals had total cholesterol levels more than 4 times those of wild type mice. Despite this striking difference in cholesterol levels, animals in both of these groups (2 and 4) had decreased aortic valve areas and similar increases in transvalvular velocities after 4 months on the hf/hc diet. These results suggest that severe hypercholesterolemia is not a pre-requisite to induce aortic valve disease in those animals. It also suggests that a combination milder hypercholesterolemia with other atherogenic risk factors such as obesity and hyperglycemia may be enough to initiate the process. Interestingly, LDLr-/- fed with normal mouse chow had mild hypercholesterolemia similar to the normal mice fed with the hf/hc diet but they did not develop any obesity nor any decrease in aortic valve area or any increases in transvalvular velocities. It seems that in LDLr-/- animals, mild hypercholesterolemia was not as effective as in normal mice to induce aortic valve abnormalities after 4 months despite the fact that LDLr-/- animals were overtly diabetic at the end of the protocol. In our model, a combination of mild hypercholesterolemia, obesity and hyperglycemia altered the aortic valve as much as a combination of severe hypercholesterolemia, overt

diabetes and obesity after 4 months. The optimal combination of atherogenic factors capable to induce the most severe and progressive aortic valve abnormalities in this animal model remains to be determined. However, our results clearly point towards a multi-factorial etiology.

Study pitfalls: The current study was short-termed and only shows the effects of the diets after 4 months. It is possible that mice in group 3 (LDLr-/- on normal diet) had mild valve abnormalities that would have become more evident after a longer follow-up. The speed of progression of the disease was not assessed and we do not know if the animals who had the most atherogenic factors (group 4 or LDLr-/- on hf/hc diet with hypercholesterolemia, obesity, diabetes) would have progressed towards a more severe aortic valve disease faster that the animals with less severe atherogenic factors (group 2 or normal mice on hf/hc diet). Blood pressure has not been measured in our animals. Some animals might have developed some degree of hypertension and hypertension has been previously linked to aortic valve disease. Finally, the current study only offers a "snap shot" view of the disease and was not designed to assess disease progression nor regression.

Conclusions:

We report for the first time the development of aortic valve disease in obese, mildly hypercholesterolemic and hyperglycemic mice resulting from an exposition to a Western high fat/high carbohydrate diet without added cholesterol. This animal model is much less extreme than previously published ones in whom severe hypercholesterolemia and a pro-calcifying agent were necessary to induce similar aortic valve abnormalities (9). We

have reproduced the early stage of aortic valve disease in this animal model in association with atherogenic risk factors more frequently encountered in humans in Western countries and sharing some similarities with the human metabolic syndrome. This model offers new research opportunities to investigators interested in the pathophysiology of aortic valve stenosis and points towards a multi-factorial etiology for this disease where hypercholesterolemia is only one factor that needs to be combined with other pro-atherogenic factors to initiate the pathologic process in the aortic valve.

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Figure Legends:

Figure 1: Aortic valve function by echocardiography *in vivo* in wt mice fed or not with a high fat/high carbohydrate diet (groups 1 and 2). A) Aortic valve area (AVA; open circles) and body weight (closed circles) evolution during the protocol in group 1 animals. B) AVA and body weight evolution during the protocol in group 2 animals. C) Maximal transvalvular velocities (cm/s) over time during the protocol in group 1 and group 2 animals. Results are presented as mean ± SEM. **: $p<0.01$ between indicated values.

Figure 2: Typical examples of maximal transvalvular aortic velocities in wt+normal diet or group 1 (top panel) and HF/HC diet or group 2 (lower panel) by continuous wave Doppler imaging after four months.

Figure 3: Leaflet thickness in group 2 (wt+hf/hc diet) vs normal controls or group 1 (wt+normal diet). Results are expressed in arbitrary units (AU) with normal leaflet thickness set at 100%.

Figure 4: Increased aortic valve leaflets thickness in group 2 animals (middle) compared to group 1 (top). Leaflets in group 2 show positive labeling for macrophages (CD68-positive cells) and foam cells (arrows). Inset shows a magnified view of the diseases leaflet in group 2. Bottom panel shows calcium deposits in an aortic valve of a group 2 animal by Von Kossa staining.

Table 1: Animal characteristics at sacrifice after 4 months of normal of HF/HC diet

Animal type	wt		LDLr-/-	
Diet	normal (Group 1) n=11	HF/HC (Group 2) n=11	normal (Group 3) n=10	HF/HC (Group 4) n=10
Body weight, g	41.2 ± 1.2	55.9 ± 2.0*	28.2 ± 0.6$^{\#}$	50.8 ± 2.3*
Heart weight, mg	148 ± 2	182 ± 8*	122 ± 2$^{\#}$	166 ± 5*
Lung weight, mg	190 ± 13	188 ± 14	147 ± 3$^{\#}$	175 ± 6
Liver weight, mg	1575 ± 84	2533 ± 209*	1062 ± 47$^{\#}$	1866 ± 192*

wt: wild type; LDLr -/-: deficient in LDL receptors. HF/HC: high fat/high carbohydrate diet. Results are expressed as mean ± SEM. *: $p<0.05$ vs. corresponding group on normal diet and $^{\#}$: $p<0,05$ vs group 1.

Table 2. Serum cholesterol, LDL, glucose and insulin in mice fed normal chow or HF/HC diet after 4 months.

Animal type	wt		LDLr-/-	
Diet	Normal (Group 1)	HF/HC (Group 2)	Normal (Group 3)	HF/HC (Group 4)
Total Cholesterol, mM (mg/dl)	2.52 ± 0.23 (97)	4.30 ± 0.41 (166)*	6.37 ± 0.26 (246) *	18.74 ± 2.26 (722)* #
LDL-Cholesterol, mM (mg/dl)	0.15 ± 0.012 (5.8)	1.18 ± 0.089 (45.5)*	3.17 ± 0.359 (122.5) *	9.15 ± 1.531 (353.1)* #
Glucose, mM (mg/dl)	14.4 ± 1.0 (260)	14.9 ± 1.7 (268)	23.6 ± 1.3 (425)*	28.6 ± 1.0*# (515)
Insulin, pM (µIU/ml)	212 ± 35 (31)	166 ± 40 (24)	15 ± 2* (2)	48 ± 14 (7)* #
Ratio Glucose/Insulin	0,09±0,021	0,16±0,055	1,88±0,327*	0,91±0,178*

Mice were fasted overnight. Data are presented as mean ± SEM (n=9-11). *: $p<0.05$ vs. group 1 and #: $p<0.05$ vs. group 3.

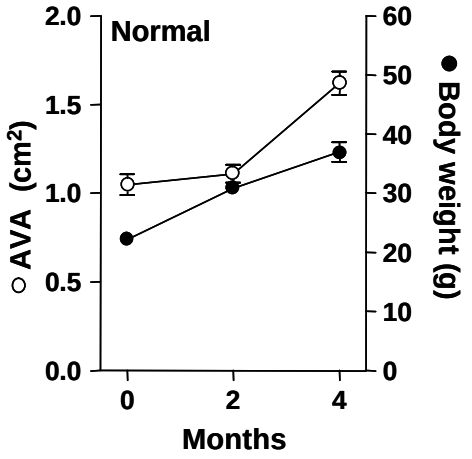
Table 3. Echocardiographic evaluation of aortic valve *in vivo* after 4 months of normal or HF/HC diet.

Animal type	wt		LDLr-/-	
Diet	Normal (Group 1) n=11	HF/HC (Group 2) n=11	Normal (Group 3) n=10	HF/HC (Group 4) n=10
AVA (mm^2)	1.62 ± 0.07	1.22 ± 0.07*	1.02 ± 0.05*	1.16 ± 0.06*
AVAi (mm^2/mg)	39.3 ± 1.2	21.8 ± 0.9*	36.2 ± 1.6	24.0 ± 1.6*#
Max. Vel. (cm/s)	83.6 ± 1.5	108.1 ± 7.4*	79.2 ± 2.1	99.8 ± 3.7*#

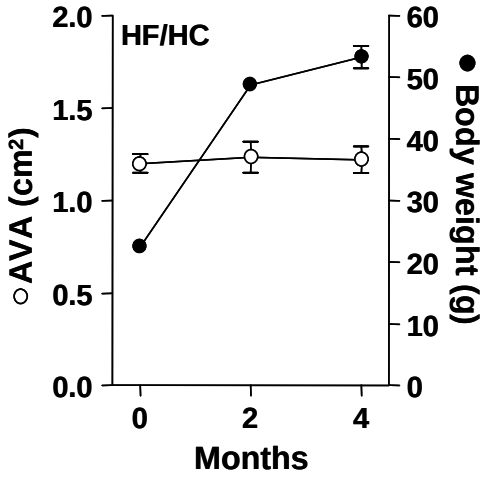
AVA: aortic valve area by continuity equation; AVAi: AVA indexed for the animal's body weight; Max Vel: maximal transvalvular velocities by continuous wave Doppler; HF/HC: high fat/high carbohydrate diet; LDLr-/-: LDL receptor deficient. Results are expressed as mean ± SEM. *: $p<0.05$ vs. group 1 and #: $p<0.05$ vs. group 3.

Figure 1

A


Normal
○ AVA (cm²)
● Body weight (g)
2.0
1.5
1.0
0.5
0.0
60
50
40
30
20
10
0
0
2
4
Months

B


HF/HC
○ AVA (cm²)
● Body weight (g)
2.0
1.5
1.0
0.5
0.0
60
50
40
30
20
10
0
0
2
4
Months

C

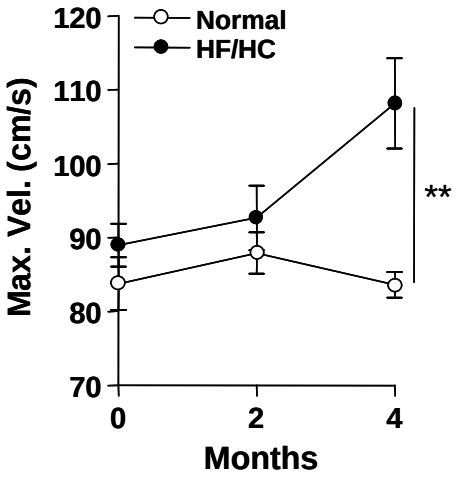

Normal
HF/HC
Max. Vel. (cm/s)
120
110
100
90
80
70
**
0
2
4
Months

Figure 2 Drolet et al.

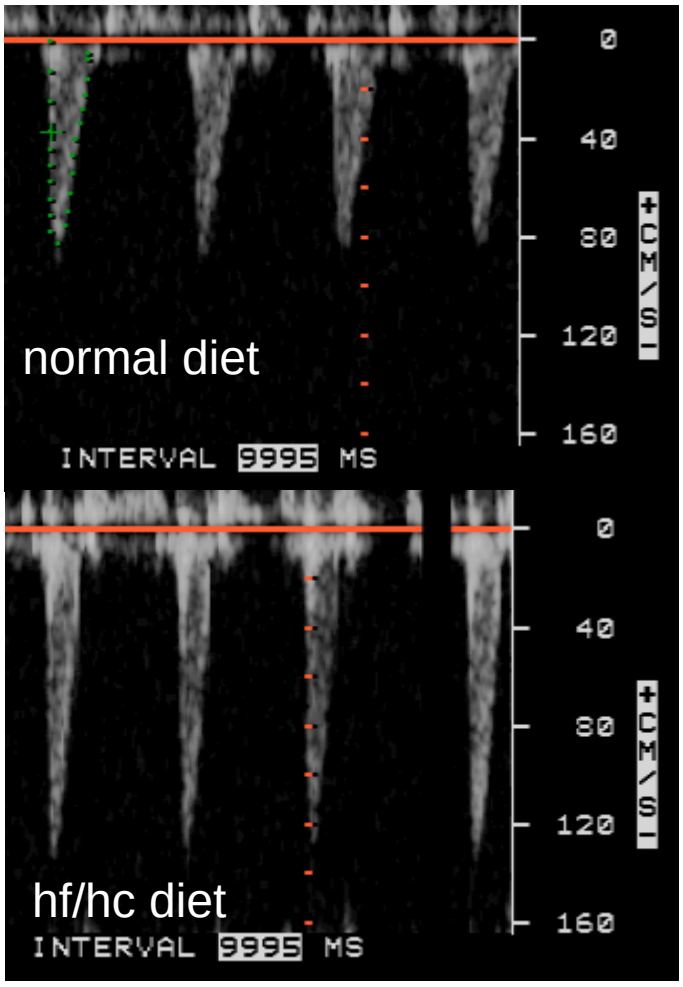
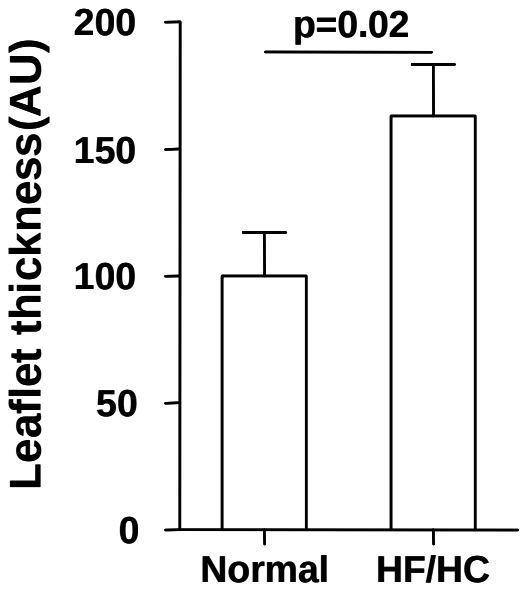
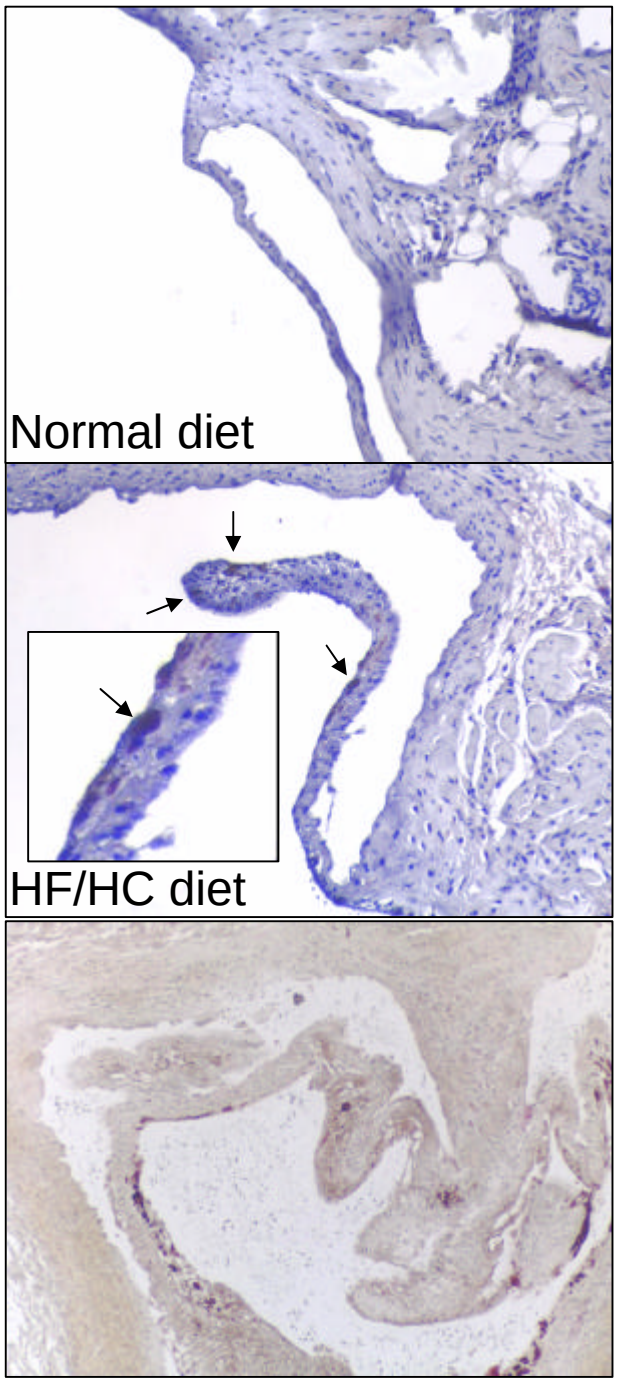


Figure 3 Drolet et al.





Drolet et al.

Figure 4