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Replacing dietary carbohydrate with protein and fat decreases the concentrations of small LDL and the inflammatory response induced by atherogenic diets in the guinea pig ☆

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Abstract

Guinea pigs resemble humans in cholesterol and lipoprotein metabolism; however, there is limited information on the vascular inflammatory response with induction of atherosclerosis in this animal model. The purpose of this study was to document a vascular inflammatory response associated with dietary-induced atherosclerosis in the guinea pig and determine the effect of replacing dietary carbohydrate with protein and fat on this response. Thirty male Hartley guinea pigs were randomly assigned to a high dietary cholesterol, high-carbohydrate (HC); a high-cholesterol, low-carbohydrate (LC) or a control (CON) diet for 12 weeks. Analysis of cytokine protein expression [interferon γ (IFN-γ), tumor necrosis factor α (TNF-α), interleukin (IL)-1β, IL-6 and GM-CSF) and m RNA expression (IFN-γ, TNF-α, IL-1β, MCP-1 and IL-8] were performed along with the measurement of cholesterol concentration in the aorta, plasma lipids and plasma low-density lipoprotein subfractions. There was a similar and significant accumulation of cholesterol in the thoracic aorta in the HC and LC groups compared to the CON group. Aortic cytokine protein expression (TNF-α, IFN-γ and IL-6) and m RNA expression (TNF-α and IFN-γ) were significantly elevated in both high-cholesterol fed groups (HC and LC) ($P<.05$) compared to the CON group. Compared to the HC group, animals fed the LC diet had reduced protein and m RNA TNF-α expression, as well as a reduced concentration of small LDL particles in the plasma. This study is the first to document a dietary cholesterol-induced vascular inflammatory response in guinea pigs that is partially regulated by the macronutrient content of the diet. Guinea pigs may be a useful animal model to evaluate the cellular and molecular components of atherosclerosis.

Keywords: Atherosclerosis; Guinea pigs; Atherogenic diets and vascular inflammation

1. Introduction

It has previously been shown that the guinea pig is the small animal model that most closely resembles humans in cholesterol and lipoprotein metabolism [1]. In contrast to other rodents and most species used for studying lipid metabolism, guinea pigs carry the majority of their plasma cholesterol in low-density lipoprotein (LDL), the atherogenic lipoprotein similar to humans, making them a unique animal model to study cholesterol and lipoprotein metabolism [1]. They are also excellent models to evaluate dietary interventions as they show aortic plaque accumulation when challenged with a hypercholesterolemic diet [1,2]. The development of early atherosclerosis has previously been demonstrated in male, female and ovariectomized guinea pigs fed hypercholesterolemic diets [3]. However, these studies all look at the progression of atherosclerosis by measurement of plaque development in the aorta and not the inflammatory cascade associated with the progression and

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development of atherosclerosis. Given that atherosclerosis is now recognized to be an inflammatory disease characterised by chronic low-grade vascular inflammation [4,5], the identification of this proinflammatory cascade in the guinea pig has the potential of allowing for this animal model to be utilised in research investigating the inflammatory mechanisms associated with atherosclerosis. The main objective of this study was to evaluate the inflammatory response induced by high dietary cholesterol in the guinea pig and the affect of manipulating the macronutrient composition of the diet. We hypothesized that a high-cholesterol diet would induce inflammation in the aorta, and the response would be attenuated by replacing carbohydrate with protein and fat.

2. Methods and materials

2.1. Animals

Thirty male Hartley guinea pigs weighing 250 to 300 g were obtained from Charles River Laboratories (Wilmington, MA, USA). At the beginning of the study, animals were randomly assigned to one of the three dietary treatments, a low-cholesterol control group (CON), a high-cholesterol/high-carbohydrate atherogenic diet group (HC) and a high-cholesterol/low-carbohydrate atherogenic diet group (LC) for 12 weeks. Guinea pigs were housed two per cage in a controlled environment at 22°C on a 12-h day/night cycle (light from 0700 to 1900 h). Diet and water were consumed ad libitum. To measure food consumption and energy intakes, the diets were weighed before and after every feeding. This study was conducted in accordance with US Public Health Service/US Department of Agriculture guidelines, and the experimental protocols were approved by the University of Connecticut Institutional Animal Care and Use Committee.

2.2. Diets

Diets were prepared and pelleted by Research Diets (New Brunswick, NJ) (Table 1). The amount of dietary cholesterol in the HC and LC groups was maintained at 0.25% in order to raise plasma cholesterol concentrations and to ensure cholesterol accumulation in the aorta and the progression of atherosclerosis [6,7]. This amount of dietary cholesterol corresponds to an absorbed amount equal to 1.5 times the daily cholesterol synthesis rates in guinea pigs [8] and is the equivalent to 1875 mg/day in the human situation. The amount of cholesterol consumed in the CON group (0.04%) was equivalent to approximately<300 mg/day in the human situation. Palm kernel oil was used as the primary fat in the diets as it is rich in lauric and myristic acids which are known to cause endogenous hypercholesterolemia in guinea pigs [3].

2.3. Plasma isolation and aortic tissue collection

Guinea pigs were anaesthetized under isoflurane vapours, and blood was obtained via cardiac puncture following a 12-h fast. Plasma samples were collected and

Table 1
Dietary composition for the CON, HC and the LC groups

Component	CON		HC		LC	
	g/100g	% Energy	g/100g	% Energy	g/100g	% Energy
Soybean protein [a]	22	23	22	23	37	34
Fat mix [b]	15.1	35	15.1	35	26	55
Corn starch/ sucrose [c]	41	42	41	42	12	11
Mineral mix [d]	8.2	-	8.2	-	8.2	-
Vitamin mix [d]	1.1	-	1.1	-	1.1	-
Cell ulose	10	-	10	-	10	-
Guar gum	2.5	-	2.5	-	2.5	-
Cholesterol	0.04	-	0.25	-	0.25	-

[a] Protein mix contains 60% casein and 40% soy protein.
[b] Fat mix contains olive oil-palm kernel oil-safflower oil (1:2:1.8). Palm kernel oil is high in lauric and myristic acids known to cause endogenous hypercholesterolemia in guinea pigs.
[c] Corn starch-sucrose ratio 1: 1.43.
[d] Mineral (S20001) and vitamin (V20001) mix were formulated to meet National Research Council requirements for guinea pigs.

protease inhibitors added to the samples (aprotonin 5 μl/ml, phenyl methyl sulfonyl fluoride1 μl/ml and sodium azide 1 μl/ml) and stored at −80°C for subsequent analysis of plasma lipids and plasma LDL subfractions. After exsanguination, the heart and aorta were removed, and the aorta was divided into three sections. The ascending aorta, aortic arch and the upper portion of the descending aorta was analysed for cytokine protein expression. The descending aorta/thoracic aorta was analysed for m RNA expression, and the lower section of the thoracic aorta was analysed for aortic cholesterol concentrations.

2.4. Plasma lipids and LDL subfractions

Plasma total cholesterol, high-density lipoprotein (HDL) cholesterol and triacylglycerol was determined by enzymatic methods [9,10]. HDL cholesterol was analysed after precipitation of apo-B-containing lipoproteins with dextran sulphate [11] using a modified method [12]. LDL subfractions and particle size was determined using nongradient polyacrylamide gel electrophoresis (Lipoprint LDL System, Quantimetrix, Redondo Beach, CA, USA). The area under the curve was calculated for each fraction, and the percentage of LDL and the mean and peak LDL particle diameter were reported. This method has been previously validated in the guinea pig [2].

2.5. Aortic cholesterol concentration

The lower section of the thoracic aorta was thoroughly cleaned of any excess tissue and fat, and aortic cholesterol concentrations were analysed using a previously established method [2]. From the cleaned tissue, aortic lipids were extracted from approximately 0.02 g of aortic tissue arch using 10 ml of chloroform:methanol (2:1) overnight at room temperature. The extraction solution was mixed with acidified water (0.5% H_2SO_4) to separate the solution into two phases, which was then

filtered by gravity filtration and the lower phase extracted with a separatory funnel. An aliquot of 200 μl, was then evaporated completely and reconstituted with 200 μl ethanol for enzymatic determination of total cholesterol.

2.6. Quantitative real-time polymerase chain reaction

Total RNA was isolated from the aortic tissue with RNeasy Fibrous Tissue Mini columns (QIAGEN, Valencia, CA, USA). Reverse transcription reactions on the isolated RNA were performed with TaqMan Reverse Transcription Reagents (Applied Biosystems, Foster City, CA, USA). Quantitative real-time polymerase chain reaction (PCR) was performed on the reverse transcribed cDNA using SYBR Green II double-stranded DNA binding dye and an Applied Biosystems 7500 Sequence Detector in accordance with previously published methods [13–16]. Guinea pig oligonucleotide primers used were the following: interferon γ (IFN-γ), forward (F) 5′-ATTTCGGTCAATGACGAGCAT-3′, reverse (R) 5′-GTTTCCTCTGGTTCGGTGACA-3′; tumor necrosis factor α (TNF-α), F 5′-CCTACCTGCTTCT-CACCCATACC-3′; R 5′-TTGATGGCAGAGAGAAG-GTTGA-3′; interleukin (IL)-1β, F 5′-CCACCAG-CCCAGGCAA-3′, R 5′-TCTACCAGCTCAACTTGGAG-GAA-3′; IL-8 (CXCL8), F 5′-GGCAGCCTTCCTGCT-CTCT-3′, R 5′-CAGCTCCGAGACCAACTTTGT-3′; MCP-1 (CCL2), F 5′-TGCCAAACTGGACCAGAGAA-3′, R 5′-CCAATGTTCAAAGGCTTTGAAGT-3′ and hypoxanthine phosphoribosyltransferase (HPRT), F 5′-AGGTGTTTATCCCTCATGGACTAATT-3′, R 5′-CCT-CCCATCTCCTTCATCACAT-3′. Currently, the only available sequence data for these guinea pig genes is cDNA; thus, it is not known whether the primers span introns. However, reverse transcriptase-negative controls were used to ensure that amplification was not due to contaminating genomic DNA. In order to ensure that only the correct gene was amplified, dissociation/melt curves for the PCR samples were also created by an additional denaturation step at 95°C for 15 s, annealing at 60°C for 20 s and a slow increase in temperature back to 95°C with a ramp time of 19 min 59 s. Results were expressed as fold induction of m RNA, which was determined from the cytokine threshold cycle values normalized for guinea pig HPRT expression and then further normalized to a calibrator value derived from pooled CON group animals.

2.7. Aortic cytokine protein expression

The aorta was thoroughly cleaned of any excess tissue and fat to ensure that no erroneous results were obtained in the assays due to any amounts of adipose tissue that could also contain cytokines. The cleaned portion of the aorta was homogenized in 3 ml of lysis buffer in a rotor-stator (VirTis, Gardiner, NY, USA) on ice for 60–90 s to ensure complete disruption of the aorta. The lysis buffer contained 10% bovine serum albumin, 0.5% TritonX-100, 1% gentamycin sulfate, 10% 100 mmol/L 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, 30 μl/ml aprotonin and 10 μl/ml 100 mM sodium orthovanadate in phosphate-buffered saline. The homogenate was stored at −80°C for measurement of aortic cytokine expression [17]. Due to the lack of commercially available or viable private sources of cytokine antibodies for the guinea pig, the cytokines IL-1β (#EMILB), IFN-γ (#EM1001), TNF-α (#EMTNFA), IL-6 (#EM2IL6) and granulocyte-macrophage colony-stimulating factor (GM-CSF) (#EMGMCSG) were determined in duplicate using commercially available mouse ELISA kits (Endogen, Rockford, IL, USA). The use of the murine antibodies was based on the mouse returning the highest sequence similarity scores against the guinea pig for several of the cytokines and chemokines using BLASTp. [18] The intra-assay coefficient of variation was 5.6%, 11%, 8.5%, 8.9% and 9.5% for IL-1β, IFN-γ, TNF-α, IL-6 and GM-CSF, respectively. Absorbances were read with a VersaMax tunable microplate reader with SoftMax Pro data reduction software (Molecular Devices, Sunnyvale, CA, USA).

2.8. Statistical analyses

Means and standard deviations were calculated for all variables using conventional methods. A one-way analysis of variance (ANOVA) was used to evaluate significant differences among the three groups of animals for the aortic cytokine protein expression, m RNA expression, plasma lipids, serum total ketone bodies, LDL particle size and aortic cholesterol concentrations. Significant main effects were further analysed using a Fisher LSD post hoc test. A criterion alpha level of $P<.05$ was used for all statistical comparisons. All data were analysed using SPSS version 11.5 (SPSS, Chicago, IL, USA).

3. Results

3.1. Animal weights and dietary intake

There were no differences in the final animal weights between the three experimental animal groups. Final weights were 798.7±72.5, 856.8±78.9 and 809.5±76.3 g for the CON, HC and LC groups, respectively. There was a significant difference in the food consumption (g/day) between groups (27.3±2.3, 28.4±0.6 and 25.0±0.9 g for the CON, HC and LC groups, respectively), although there was no difference in the average daily energy intake due to the varying energy densities between the diets (3.88 kcal/g for the CON and HC groups and 4.33 kcal/g for the LC group). There was also a significantly great intake of palm kernel oil in the LC group compared to the other two groups, which corresponded to the greater percentage of total energy obtained from fat for this group (55%).

3.2. LDL particle size and subfractions

Plasma lipid responses to these diets have been reported elsewhere [19]. Plasma total and LDL cholesterol were higher in the high cholesterol groups compared to the control

Table 2
LDL subfractions (percentage) and LDL particle size in the guinea pigs fed the CON, HC or LC diets after 12 weeks

	CON	HC	LC
LDL 1 (%) (27.7 nm)	18.5±6.0[a]	13.2±4.7[b]	14.9±4.3[a]
LDL 2 (%) (26.1 nm)	9.9±5.8	11.0±2.5	14.4±5.9
LDL 3 (%) (24.5 nm)	0.5±0.9[a]	4.9±2.7[b]	2.0±1.9[a]
LDL 4-7 (%) (23-18.7 nm)	0.0±0.0[a]	3.2±2.9[b]	0.4±0.9[a]
Mean diameter (nm)	27.1±0.3[a]	26.2±0.7[b]	26.8±0.6[a]
LDL phenotype (no. of guinea pigs A)	8/10	4/10	7/10

Values are mean±S.D. for n=10 guinea pigs per group. Values in parentheses indicate the mean particle size for that fraction. Data was analysed with a one-way ANOVA. Values in the same row with different superscript are significantly different as determined by one-way ANOVA.

group. As indicated in Table 2, guinea pigs fed the HC diet had lower concentrations of the larger LDL, compared to both the control and the LC group. The LC group resulted in lower concentrations of the smaller LDL subfractions and a larger mean LDL size compared to the HC group. Also, more guinea pigs in the LC group were classified as pattern A, compared to the HC group, indicating a major effect of macronutrient composition of LDL particle distribution.

3.3. Aortic cholesterol concentration

The measurement of cholesterol accumulation in the thoracic aorta showed that both the HC and LC groups had a significantly greater accumulation of total cholesterol compared to the CON group (Fig. 1).

3.4. mRNA expression for guinea pig specific cytokines and chemokines

There was a significant increase in both the HC and LC groups for the expression of IFN-γ m RNA (HC: ↑4.5-fold; LC: ↑3.3-fold) and TNF-α m RNA (HC: ↑3.1-fold; LC: ↑1.4-fold) compared to the CON group (Fig. 2). An interesting finding was that low carbohydrate reduced the expression of TNF-α when compared to the HC group. There were no other significant differences for IL-1β, IL-8 or MCP-1 for any of the groups.

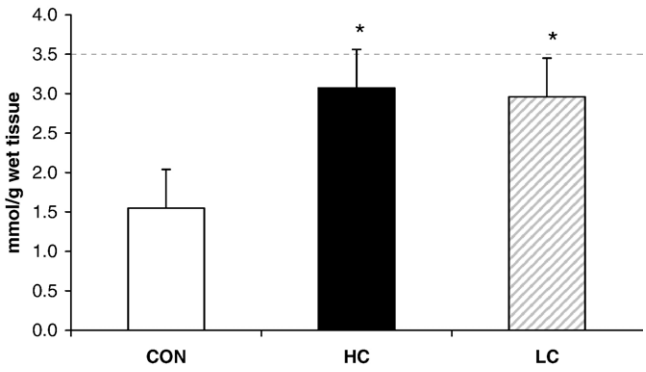

Fig. 1. Total cholesterol accumulation in the thoracic aorta (mmol/g wet tissue) in the three experimental animal groups after the 12-week atherogenic and control diets (mean <.05 compared to CON group.

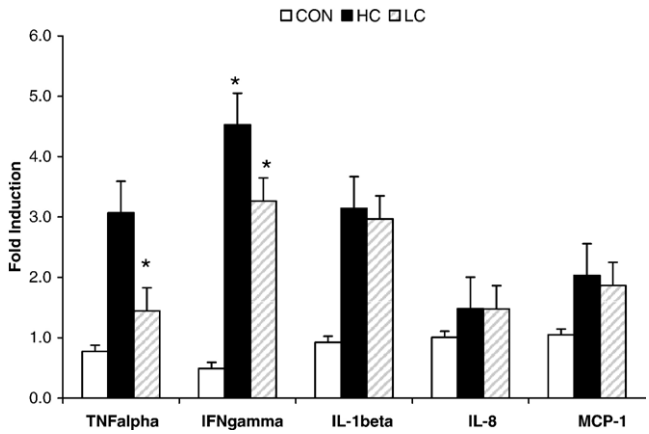

Fig. 2. m RNA expression of the cytokines IFN-γ, TNF-α, IL-1β and the chemokines IL-8 and MCP-1 in the thoracic aorta between the three experimental animal groups after the 12-week atherogenic and control diets (mean±S.E.M.). Fold induction was determined from the threshold cycle values normalized for guinea pig HPRT and then further normalized to the value derived from pooled low-cholesterol group samples. *P<.05 compared to CON group. †P<.05 compared to HC group.

3.5. Aortic cytokine protein expression

Normalized cytokine protein optical density (OD) measurements for the HC and LC groups were expressed as a percentage relative to the OD obtained for the CON group. BLASTp [18] was used to query the amino acid sequence similarity for all available sequences between the mouse (*Mus musculus*) and guinea pig (*Cavia porcellus*) (TNF-α 81%, IL-1β 70%, IFN-γ 40% and GM-CSF 43%). There is no sequence currently available for IL-6 in the guinea pig, so no information is displayed for this cytokine. High cholesterol increased the expression for all cytokines compared to the low cholesterol group (CON group) (Fig. 3). The restriction in dietary carbohydrate reduced the concentration of TNF-α and IL-6 compared to the HC group demonstrating a positive effect of low carbohydrate diets on inflammation.

4. Discussion

The primary aim of this study was to quantify the vascular inflammatory response associated with dietary-induced atherosclerosis in the guinea pig. The results clearly showed a pro-inflammatory response at the level of m RNA and protein expression associated with high dietary cholesterol in this guinea pig model. To our knowledge, this study is the first study to confirm that guinea pigs demonstrate an increased vascular inflammatory response after the intake of an atherogenic diet. We further showed that the inflammatory response is responsive to the background macronutrient composition; specifically, a diet that replaced carbohydrate with protein and fat resulted in reduced protein and m RNA expression of TNF-α and reduced protein expression for IL-6.

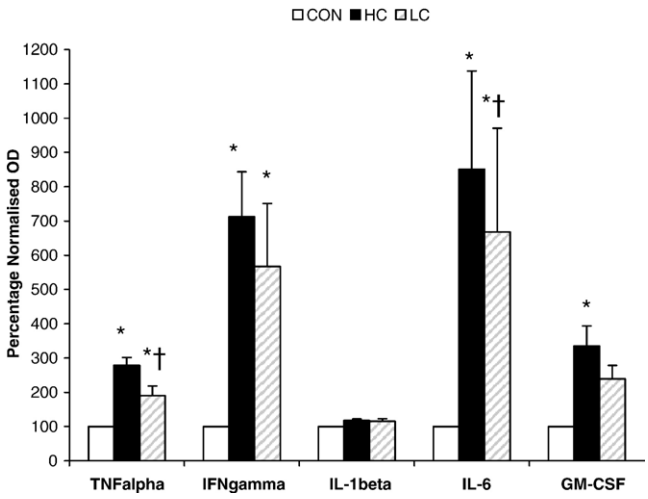

Fig. 3. Normalised OD measured with murine antibodies for the cytokines TNF-α, IFN-γ, IL-1β, IL-6 and GM-CSF in the aortic arch between the three experimental animal groups after the 12-week atherogenic and control diets (mean±S.E.M.). Normalized ODs for the HC and LC groups were expressed as a percentage against the CON group. *P<05 compared to CON group. †P<05 compared to HC group.

The significantly greater expression of TNF-α and IL-6 in the HC versus the LC groups may be due to the significantly greater percentage of small dense LDL (sdLDL) particles in the plasma in this group. Small LDL particles are thought to be more susceptible to oxidative modification and subsequent uptake by scavenger receptors on activated macrophages leading to the development of lipid-engorged foam cells. These foam cells contribute to the secretion of many of the proinflammatory cytokines that then contribute to the inflammatory response in atherosclerosis [20]. TNF-α expression was found to be significantly lower in the LC group compared to the HC group. Ares et al. [21] demonstrated in vitro that macrophages incubated with acetylated LDL (acLDL) resulted in a decrease in nuclear factor κB (NF-κB) activity and TNF-α secretion. This finding in vitro of decreased inflammatory cytokines in cholesterol laden macrophages has been verified in many other studies [22–25]. This provides indirect support for the concept that LDL uptake results in down-regulation of the inflammatory functions of monocytes/macrophages. It has therefore been suggested that the inhibition of NF-κB by modified lipoproteins (oxidizedLDL and acetylatedLDL) could attenuate the inflammatory response to these lipids, but it could also make macrophages cells more susceptible to apoptosis [26,27]. Based on these findings [22–25], the reduction in TNF-α and IL-6 expression in the LC group may have been caused by a down-regulation in the transcription of these genes by NF-κB. However, as measurement of the lipoprotein composition of the macrophages resident in the atherosclerotic lesions was not possible in this study, this conclusion must be presented as speculation. Further investigations are necessary to evaluate this finding in vivo in guinea pigs.

Both IFN-γ protein and m RNA expression were significantly increased after the intake of both high-cholesterol diets. Because IFN-γ is known to directly activate macrophages and indirectly activate lymphocytes by increasing antigen presentation, this cytokine plays a major role in perpetuating the proatherogenic environment within lesion-affected vessels. In human atherosclerosis, it is known that IFN-γ levels are significantly increased within atherosclerotic vessels [4]. Increased expression of IFN-γ up-regulates IL-1β, TNF-α and MCP-1 production along with the transcription of genes encoding GM-CSF [28,29]. It also appears that exogenous IFN-γ administration potentiates lesion formation in hypercholesterolemic mice [30]. These findings led Gupta et al. [31] to suggest that IFN-γ can potentiate atherosclerosis through both local effects in the arterial wall and systemic effects on plasma lipoproteins [32]. Buono et al. [33] has shown direct evidence that IFN-γ influences atherosclerosis development in LDLreceptor$^{-/-}$ mice independent of changes in blood lipoprotein profiles. The findings in this study agree with that by Buono et al., as even though there were significant differences in the plasma lipoproteins between the two high-cholesterol groups, there were no differences between either the protein or m RNA expression for IFN-γ.

There was a significant increase in GM-CSF protein expression for the HC group but not for the LC group. GM-CSF has been shown to have proinflammatory effects on monocyte activation [34] and T cell function [35]. In the murine model, GM-CSF is critical for the development of a number of inflammatory and autoimmune diseases [36]. However, the *in vivo* analysis of the mode of action of GM-CSF as a proinflammatory cytokine has been hampered by the difficulty in isolating cell populations in sufficient quantity and purity from atherosclerotic lesions. The reason for the significant increase in GM-CSF concentrations for the HC group and lack of an increase for the LC group in this study is not known. Though, it may be the significantly lower expression of TNF-α in the LC group that may have down-regulated the transcription of GM-CSF.

As expected, both high-cholesterol groups had significantly higher plasma LDL concentrations due to the hypercholesterolemic effect of the atherogenic diets. The measurement of LDL subfractions showed a significant increase in the LDL subfractions 4-7 in the HC group. These subfractions correspond to the sdLDL particles (23–18.7 nm). The intrinsic properties of sdLDL particles have been suggested to be biologically responsible for increasing the risk of developing CAD [37–39], with numerous studies reporting that the presence of sdLDL particles is associated with a more than threefold increase in risk of coronary artery disease [38–40]. sdLDL also penetrate the arterial wall more easily [41] and have a higher capacity to bind to intimal proteoglycans [42,43], all properties that are associated with greater atherogenecity.

A significant increase in the thoracic aortic total cholesterol concentration in the HC and LC groups was

also found, suggesting a significant aggregation of lipoproteins into the arterial wall. It is important to mention that the measurement of aortic cholesterol accumulation was performed in the descending thoracic aorta. Measurements obtained in this study are similar to those that have been obtained in other guinea pig studies that have measured the cholesterol accumulation in the aortic arch [2]. This finding is important as it shows that guinea pigs demonstrate plaque accumulation along the thoracic aorta similar to what is seen in humans. This is dissimilar to the distribution of lesions in other rodents models where plaque accumulation is generally limited to the aortic sinus [44].

Evidence from other experimental animal models of atherosclerosis indicates that the initiation of a high-cholesterol atherogenic diet results in the rapid activation of the adhesion molecule cascade and proinflammatory cytokines. Consequently, our results provide merit for further use of the guinea pig as a model of atherosclerosis encompassing both aspects of cholesterol and lipoprotein metabolism and also the ensuing immune system activation. However, it is important to note that much more work is needed to quantify in detail the exact cellular and molecular immune signalling pathways that act in response to dietary-induced atherosclerosis in this model. It will be crucial for further studies to document not only the localization of the atherosclerotic lesions in this animal model but also the immune components present in lesions to gain a wider picture of the cellular and molecular mechanisms involved in the development of atherosclerosis. As a result, the continued use of the guinea pig will greatly depend upon the further studies verifying these findings, and future studies should document not only the lesion location but severity of lesion development, and whether they develop advanced lesions susceptible to thrombosis.

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