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Therapeutic Intranasal Vaccine HB-ATV-8 Prevents Atherogenesis and Non-alcoholic Fatty Liver Disease in a Pig Model of Atherosclerosis

Roxana Gutiérrez-Vidal,[a] Blanca Delgado-Coello,[a] Kevin Manuel Méndez-Acevedo,[a,b] Sandra Calixto-Tlacomulco,[a] Salvador Damián-Zamacona,[c] and Jaime Mas-Oliva[a]

[a]*Departamento de Bioquímica y Biología Estructural, Instituto de Fisiología Celular, Universidad Nacional Autónoma de México, Ciudad de México, México*
[b]*Max Delbrück-Centrum für Molekulare Medizin, Berlin, Germany*
[c]*Bioextracto, S.A. de C.V., Ciudad de México, México*

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Background and Aims. Atherosclerosis as an inflammatory disease involved in the etiology of cardiovascular disease worldwide, in our days demands an array of different therapeutic approaches in order to soon be able to visualize an effective prevention. Based on an immunotherapeutic approach, we designed a non-invasive vaccine (HB-ATV-8), contained in a micellar nanoparticle composed of lipids and a peptide segment derived from the C-terminus of the cholesterol-ester transfer protein (CETP). Now we extend our successful proof of concept from the rabbit to a porcine model and investigated its effect in an attempt to undoubtedly establish the efficacy of vaccination in a model closer to the human.

Methods. A preclinical trial was designed to study the efficacy of vaccine HB-ATV-8 in pigs (Large White × Landrace). Male experimental animals were fed with standard diet (control), high fat diet (HFD) or the same HFD but treated with HB-ATV-8 (HFD + Vaccine) applied nasally for up to 7 months. All biochemical and enzymatic analyses were performed in peripheral venous blood and thoracic aorta and liver samples examined using conventional, two-photon excitation and second harmonic generation microscopy to identify atherosclerotic and hepatic lesions. mRNA concentrations for *KLF2*, *ACTA2*, *SOD1, COL1A1* genes and protein levels for PPARα and ABCA1 were quantified in aorta and liver respectively using qPCR and Western blot analysis.

Results. The administration of vaccine HB-ATV-8 induced anti-CETP IgG antibodies and reduced atherosclerotic and hepatic lesions promoted by the high fat diet. In addition, plasma triglyceride levels of vaccine treated pigs fed the HFD were similar to those of control group, in contrast to high concentrations reached with animals exclusively fed with HFD. Moreover, HFD promotes a tendency to decrease hepatic PPARα levels and increase in aorta gene expression of *KLF2*, *ACTA2*, *SOD1 and COL1A1*, while vaccine application promotes recovery close to control values.

Conclusions. Vaccine HB-ATV-8 administration constitutes a promissory preventive approach useful in the control of atherogenesis and fatty liver disease. The positive results obtained, the non-invasive characteristics of the vaccine, the simple design employed in its conception and its low production cost, support the novelty of this therapeutic strategy designed to prevent the process of atherogenesis and control the development of fatty liver disease.

Key Words: Atherogenesis, NAFLD, CETP, Nasal therapeutic vaccine, Porcine model.

Address reprint requests to: Jaime Mas-Oliva, Departamento de Bioquímica y Biología Estructural, Instituto de Fisiología Celular, Universidad Nacional Autónoma de México, Apartado Postal 70-243, 04510 Ciudad de México, México; Phone: (+52) (55) 5622-5584; FAX: (+52) (55) 5622-5611; E-mail: jmas@ifc.unam.mx

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Introduction

Atherosclerosis is considered an inflammatory disease of the arterial wall that leads to cardiovascular disease (CVD), a common cause of death in the world according to WHO (1). Epidemiological studies have indicated that among the main risk factors associated with atherosclerosis there is an increased plasma level of low density lipoproteins cholesterol (LDL-C) associated to a decreased plasma concentration of high density lipoprotein cholesterol (HDL-C) (2−4). Nevertheless, the relationship between the concentration of the different plasma lipoproteins and its probability to develop atherosclerosis, is still unclear (5,6). Considering that a reduction of CVD nowadays should be directed towards prevention rather than treatment, there has been an important effort to reinforce measures mainly directed to a change in lifestyle and eating habits in order to impede the onset of atherosclerosis (7,8). Although this approach should be considered central in all prevention efforts, it has contributed little to the prevention of CVD in most countries of the western world (9,10).

From the pharmacologic point of view, although statins for years have offered a way to decrease the plasma concentration of LDL-C by acting upon the biosynthesis pathway of cholesterol, this approach has not been successful enough based on the fact that the number of deaths related to CVD associated with atherosclerosis continues to increase (11,12). Therefore, treatment of atherosclerosis exclusively based on a cholesterol lowering therapy still has to prove a significant efficacy in reducing CVD.

In consequence, considering this process is characterized by the accumulation of fat in the intima of arteries where innate and adaptive immunity play an important role in the process of atherogenesis (13,14), modulation of the immune response has attracted attention as a strategy for its prevention and treatment helping to restore the homeostasis of lipid metabolism and counteract an inflammatory state (15,16). In this sense, the study of several possibilities that contemplate potential proteins have been identified for their ability to decrease morbidity and mortality associated with the progression of atherosclerosis (17,18). Among the diverse strategies employed, the cholesteryl-ester transfer protein (CETP) has been studied as a potential therapeutic target (19−21). CETP promotes the mobilization of cholesteryl-esters, triglycerides and phospholipids between HDL and lipoproteins containing apolipoprotein B (ApoB) (LDL and VLDL) (22). Corresponds to a 66−74 kDa plasma glycoprotein that is mainly expressed in the liver and secreted into the bloodstream where it becomes associated to lipoproteins (23). Population studies have shown that a decreased concentration and/or reduced CETP activity are associated with increased HDL-C levels (23−25), and although several studies present a less clear correlation (26,27), most of them show that subjects presenting a low plasma CETP concentration show a lower probability to develop a cardiovascular event. In support of these findings, there are several reports indicating that a group of Japanese subjects while lacking CETP, present high HDL-C levels, low LDL-C levels and a low incidence of CVD (21,28).

The nasal administration of vaccine HB-ATV-8 composed of a micellar nanoparticle preparation has been designed as an immunotherapy to decrease CETP activity *in vivo*. Vaccine nanoparticles incorporate a 12 amino acid synthetic peptide corresponding to the C-terminal domain of CETP added of an N-terminal cysteine and a key mixture of lipids including caldarchaeol (29,30). As previously reported by us, the use of lysophosphatidylcholine allows the peptide to be kept in a key α-helical conformation (31,32), achieving at the same time structural stability and immunogenicity (33,34). Therefore, here we present an easy, cheap and efficient way to produce immunogenicity by applying a nanoparticulate preparation on the surface of the nasal mucosa.

Previous work from our group employing cholesterol-fed rabbits demonstrated that the nasal administration of vaccine HB-ATV-8 significantly reduces atherosclerotic lesions in the aorta, decreasing in parallel the presence of non-alcoholic fatty liver disease (NAFLD) in association with liver fibrotic lesions (16). This data might be considered important taking into account that CVD has been shown to be the leading cause of death in patients with NAFLD (35,36).

Now, before initiating the clinical phases for vaccine HB-ATV-8 to define safety and efficacy, we have extended our proof of concept to an animal model closer to human and studied the effect of therapeutic vaccine HB-ATV-8 in the pig as the experimental animal. Since the pig closely resembles the human in many traits including its anatomy, physiology, biochemistry and ultimately lifestyle (37−39), it has been considered for years an excellent non-primate model to study atherosclerosis. Therefore, the present study has been conducted for seven months employing pigs in an attempt to closely resemble conditions for the development of atherogenesis and fatty liver in the human.

Materials and Methods

Vaccine HB-ATV-8

Vaccine HB-ATV-8 (Patents US9539312, MX347400 B) contains as immunogen a synthetic peptide that corresponds to the carboxy-end amino acids H486−S496 of CETP (29,30). This peptide conjugated into a micellar nanoparticle system includes three of the four key residues that support lipid binding and transfer capacity (40). Micelles are composed of lipids derived from the cell membrane of *Thermus aquaticus*, mainly caldarchaeol. L-α-Phosphatidylcholine (PC) and 1-lauroyl-2-hydroxy-sn-glycero-3-phosphocholine (lyso-C_{12}PC) (31,32). These components

have been shown function as humoral adjuvants promoting a strong cytotoxic T-cell immune response characterized by a long-term memory (41–43).

Experimental Animal Procedures

The present study includes fifteen castrated male pigs (Large White x Landrace) housed at the Specific Pig facility (Barcelona, Spain). All animal procedures and experiments were performed in accordance with the Guide for the Care and Use of Laboratory Animals (NIH) and approved by the Deparment d'Agricultura, Ramaderia, Pesca, Alimentació i Medi Natural, de la Generalitat de Calalunya, and the Specific Pig Ethics Committee. The qualified staff of Specific Pig fed the animals, nasally administered the vaccine, collected blood samples, monitored their well-being, sacrificed the animals and collected tissues at the end of the experiment.

Pigs were housed at 10–24°C on a 12:12 h light/dark cycle in 3.32 m^2 pens (3–4 animals/pen) with a dry, non-toxic, absorbent and pathogen-free bed. The animals had free access to water and fed once daily. At the beginning of the protocol the average weight of animals corresponded to 40 kg. The study was based on three experimental groups: *Control group* (CT, $n = 3$) corresponds to pigs fed a standard diet (Porcs creixement 1, Pinallet) composed of 15.8% calories from protein, 3.5% from carbohydrates, and 3.7% from fat were kept for 4 ($n = 1$) and 7 months ($n = 2$). *High-Fat Diet group* (HFD, $n = 6$) corresponds to pigs fed a high-fat atherogenic diet (HFD) (SDS, 824100 Porcine Western, Dietex, France) for 4 ($n = 4$) and 7 months ($n = 2$). The diet contained 44% calories from fat (15% lard and 2% cholesterol), 16% calories from protein, and 40% calories from carbohydrates. *High-Fat Diet + Vaccine HB-ATV-8 group* (HFD + Vaccine, $n = 6$) corresponds to pigs fed the high-fat atherogenic diet that received vaccine HB-ATV-8 (300 µg) nasally administered twice a week for 4 ($n = 4$) and 7 months ($n = 2$). Blood samples from each experimental animal were taken every four weeks, and the experiment followed in two stages (4 and 7 months) as mentioned above where a certain number of animals at these specific times were anesthetized by intramuscular administration of ketamine (10–33 mg/kg) and midazolam (0.3–0.5 mg/kg), and euthanized with a sodium pentobarbital overdose (50–80 mg/kg, IV). Figure 1 summarizes the experimental protocol followed.

Plasma Lipid Profile and Enzyme Activity Measurements

Fifteen milliliters of peripheral venous blood were collected from the cranial vena cava at the beginning of the study and in a monthly basis till the end of the experiment. Serum and plasma-EDTA samples were used for biochemical and enzyme activity measurements, and the remaining biological material stored at –80°C until analysis. Zoologic Veterinaris laboratory (Barcelona, Spain) performed all lipid and enzymatic measurements. The lipid profile was measured by enzymatic methods following the manufacturers' instructions (Gernon platform, Ral, Spain). Plasma enzyme activity for aspartate aminotransferase, alanine aminotransferase, alkaline phosphatase and total protein concentration were measured by standardized and commercially available colorimetric assays (Gernon platform).

Histological Examination

At the time of euthanasia, the thoracic aorta and liver samples from the right lobule were collected and a portion of these samples fixed in 10% formaldehyde. Samples were embedded in paraffin and stained with hematoxylin-eosin (H&E) and Masson's trichrome stain. The remaining tissue was frozen in liquid nitrogen and stored at −80°C until further analysis. Histological results were obtained by reviewing five different optical fields of view where percentages represent the frequency of the characteristics observed within each group.

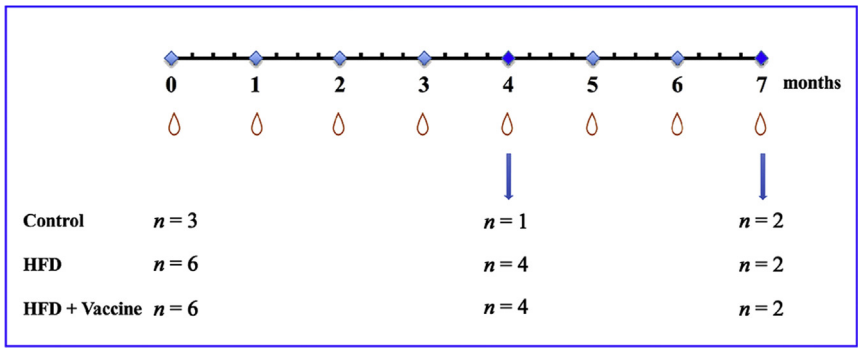

Figure 1. Experimental design. Control group (CT, $n = 3$); one animal from this group sacrificed at month 4 and the remaining two at month 7. High-fat diet group (HFD, $n = 6$); four animals from this group sacrificed at month 4 and the remaining two at month 7. High fat diet + vaccine treatment group (HFD + Vaccine, $n = 6$); four animals from this group sacrificed at month 4 and the remaining two at month 7. Blood samples were taken every month. HFD + vaccine group were administered with vaccine HB-ATV-8 twice a week.

Classification of atherosclerotic lesions was performed according to the guidelines of the American Heart Association (AHA) (44). The six aortic lesion types defined by the AHA are: (type I) isolated macrophage foam cells; (type II) intracellular lipid accumulation; (type III) type II changes and small extracellular lipid pools; (type IV) type III changes and presence of an extracellular lipid core; (type V) presence of a lipid core and a fibrotic layer or multiple lipid cores and fibrotic layers, areas of calcification; (type VI) all previous findings plus the presence of areas of hematoma/hemorrhage and thrombus.

Liver sections were evaluated considering histopathological characteristics for NAFLD: steatosis, inflammation, ballooning, and fibrosis (45,46) and data reported as presence or absence of these characteristics.

Two-photon Excitation Microscopy and Second-harmonic Generation Microscopy

Two-photon imaging was performed using a galvanometer-based scanning system LSM 710-Zeiss and a Ti: sapphire laser (Coherent) employing a wavelength of 850 nm (2.5% of laser power) for H&E stained aorta and liver slides. Second-harmonic generation imaging was performed using a wavelength of 900 nm (12% of laser power) and a BP 420−480 nm filter (Zeiss). Images were acquired using water immersion objectives; C-Apochromat 10x and W Plan-Apochromat 20x, with a 40x optical zoom for liver slices.

Anti-CETP Titer Determination

IgG antibody titer for CETP was measured by ELISA. Briefly, plates were coated with CETP synthetic peptide H486-S496, blocked and incubated with pig serum (1:100), a goat horseradish peroxidase-conjugate anti-pig IgG, and tetramethylbenzidine. OD was measured at 450 nm.

Protein Expression Analysis

Liver samples were homogenized in cold RIPA buffer (Thermo Fisher Scientific, MA, USA) supplemented with protease inhibitors (Roche, Rotkreuz, Switzerland). Total protein concentration (serum and liver extracts) was measured using the DC Protein Assay (Bio-Rad, CA, USA). Serum proteins were separated by SDS-PAGE using 8% gels and transferred to PVDF membranes (Immobilon-P, Millipore, MA, USA) to be analyzed by Western blot. Blots were incubated overnight with rabbit anti-CETP (Thermo Fisher Scientific, MA, USA) or mouse anti-ACTB antibodies (Santa Cruz Biotechnology, CA, USA). Also, membranes for liver extracts were incubated with mouse anti-PPARα, anti-ABCA1 and anti-GAPDH antibodies (Santa Cruz Biotechnology, CA, USA). Immunoreactive proteins were visualized with the Immobilon Western Chemiluminiscent HRP reagent (Millipore, MA, USA). Blots were quantified using the Image J software (http://rsb.info.nih.gov/ij/) and results presented as the CETP/ACTB (β-actin) ratio for serum protein and PPARα or ABCA1/GAPDH ratio for liver extracts.

Quantitative PCR Measurements

Total RNA was extracted from the thoracic aortas with Trizol reagent (Thermo Fisher Scientific, MA, USA). 1 µg of total RNA was used to synthesize cDNA using the iScript cDNA synthesis kit (Bio-Rad, CA, USA) and cDNA diluted to perform qPCR experiments. Expression of Krüppel-like factor-2 (*KLF2*), smooth muscle alpha (α)-2 actin (*ACTA2*), superoxide dismutase 1 (*SOD1*), collagen type I alpha 1 (*COL1A1*) and the housekeeping gene *ACTB* was determined by qPCR using the PowerUp Sybr Green Master Mix 2X (Applied Biosystems, CA, USA) on an ABI PRISM 7000 Sequence Detection cycler. Primer sequences are reported as supplementary material as well as relative levels of mRNA calculated as $2^{-\Delta\Delta Ct}$ (Supplementary Table 1).

Statistical Analysis

Data are expressed as mean±S.E.M. Statistical differences among study groups were calculated using ANOVA or Kruskal-Wallis tests, depending on the distribution of variables. p values ≤ 0.05 were considered significant. Analysis of data was performed with the SPSS v20 program (SPSS, Chicago, USA).

Results

The nasal administration of vaccine HB-ATV-8 induced the formation of anti-CETP IgG antibodies in the group of pigs fed a HFD where the rise in antibody titer became evident after the fourth month of treatment and remained at a high level until the end of the study (Figure 2A). Although the CETP serum concentration was kept at the same level throughout the study in the three groups of experimental animals (Figure 2B), the body weight of pigs fed a HFD was higher compared to those fed a standard diet regardless of whether they received the treatment or not (Figure 2C).

Since it is known that CETP is able to modify the serum level of the different types of lipoproteins by mobilizing cholesteryl-esters and triglycerides, we evaluated if the administration of vaccine HB-ATV-8 altered the serum level for these lipids. As expected, it was found that HFD fed pigs showed a steady increase of total cholesterol, LDL, HDL cholesterol and triglycerides (Figure 3). Interestingly, the only parameter that was significantly modified by the administration of vaccine HB-ATV-8 corresponds to triglycerides that remained at levels similar to the control group (Figure 3D). The serum level for total proteins and

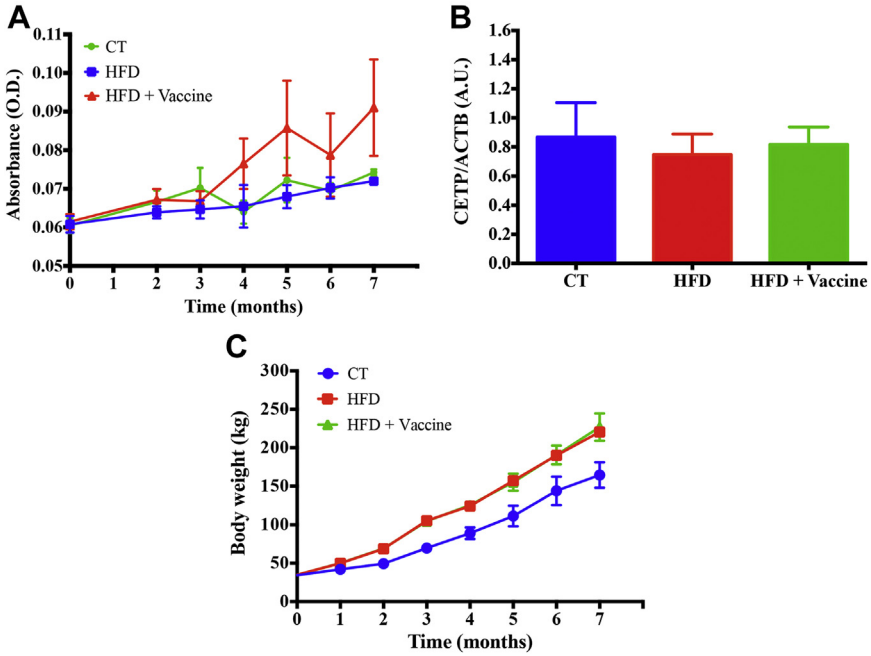

Figure 2. Effect of vaccine HB-ATV-8 administration on IgG anti-CETP antibody production and CETP plasma concentration. (A) Plasma IgG anti-CETP level of pigs from the three experimental groups. (B) Serum CETP concentration from the three experimental groups using Western blot analysis (data expressed as CETP/ACTB ratio). (A) and (B) Data represented as mean ± S.D. (C) Body weight. Control group (CT, $n = 3$); high-fat diet group (HFD, $n = 6$); and high-fat diet group + vaccine (HFD + Vaccine, $n = 6$). Data represented as mean ± S.E.

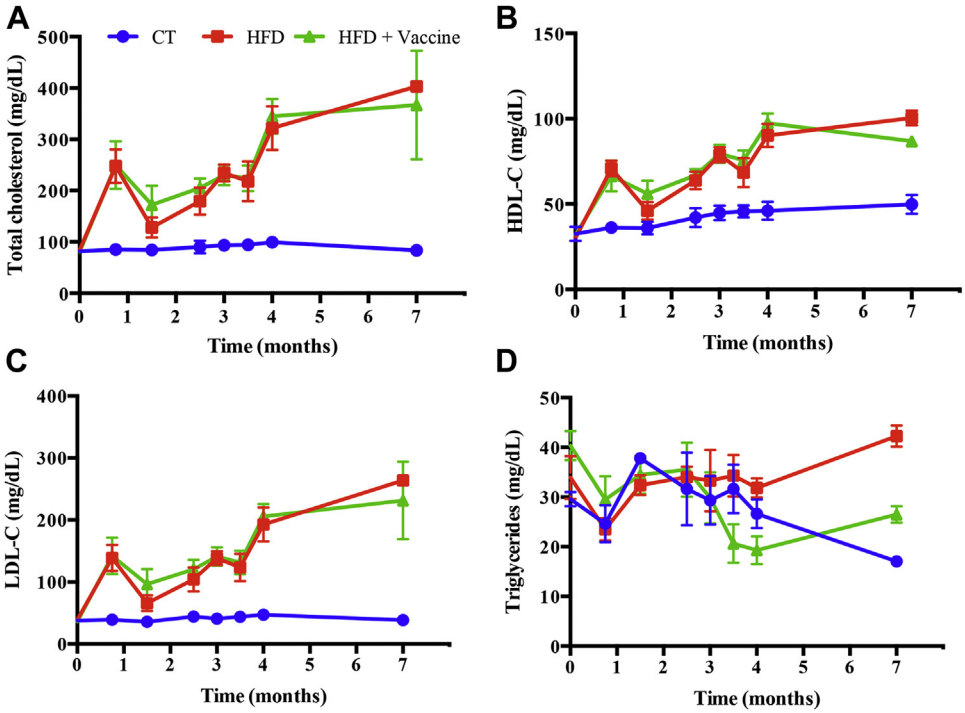

Figure 3. Effect of vaccine HB-ATV-8 administration on serum lipid profiles during the 7 months of treatment. (A) Total cholesterol; (B) HDL-C; (C) LDL-C, and (D) Triglycerides. Control group (CT, $n = 3$); one animal from this group sacrificed at month 4 and the remaining two at month 7. High fat diet group (HFD, $n = 6$); four animals from this group sacrificed at month 4 and the remaining two at month 7. High fat diet + vaccine treatment group (HFD + Vaccine, $n = 6$); four animals from this group sacrificed at month 4 and the remaining two at month 7. Data represented as mean ± S.E.

the activity for liver function enzymes such as transaminases and alkaline phosphatase remained stable without statistically significant differences among groups (Supplementary Table 2).

At the end of the experiment, thoracic aorta and liver samples were prepared and examined by conventional light microscopy together with two-photon excitation and second harmonic generation microscopy. Figure 4 shows representative images for samples of the thoracic aorta of pigs belonging to each one of the three experimental groups studied. As expected, aortas from pigs fed the standard diet showed a normal vessel structure. In contrast, pigs fed a HFD show lipid droplets and foam cells in the media and the intima layers of the aorta and an increase of vascular smooth muscle cells (VSMC) in the intima layer (Figure 4A and B) and present type II and III atherogenic

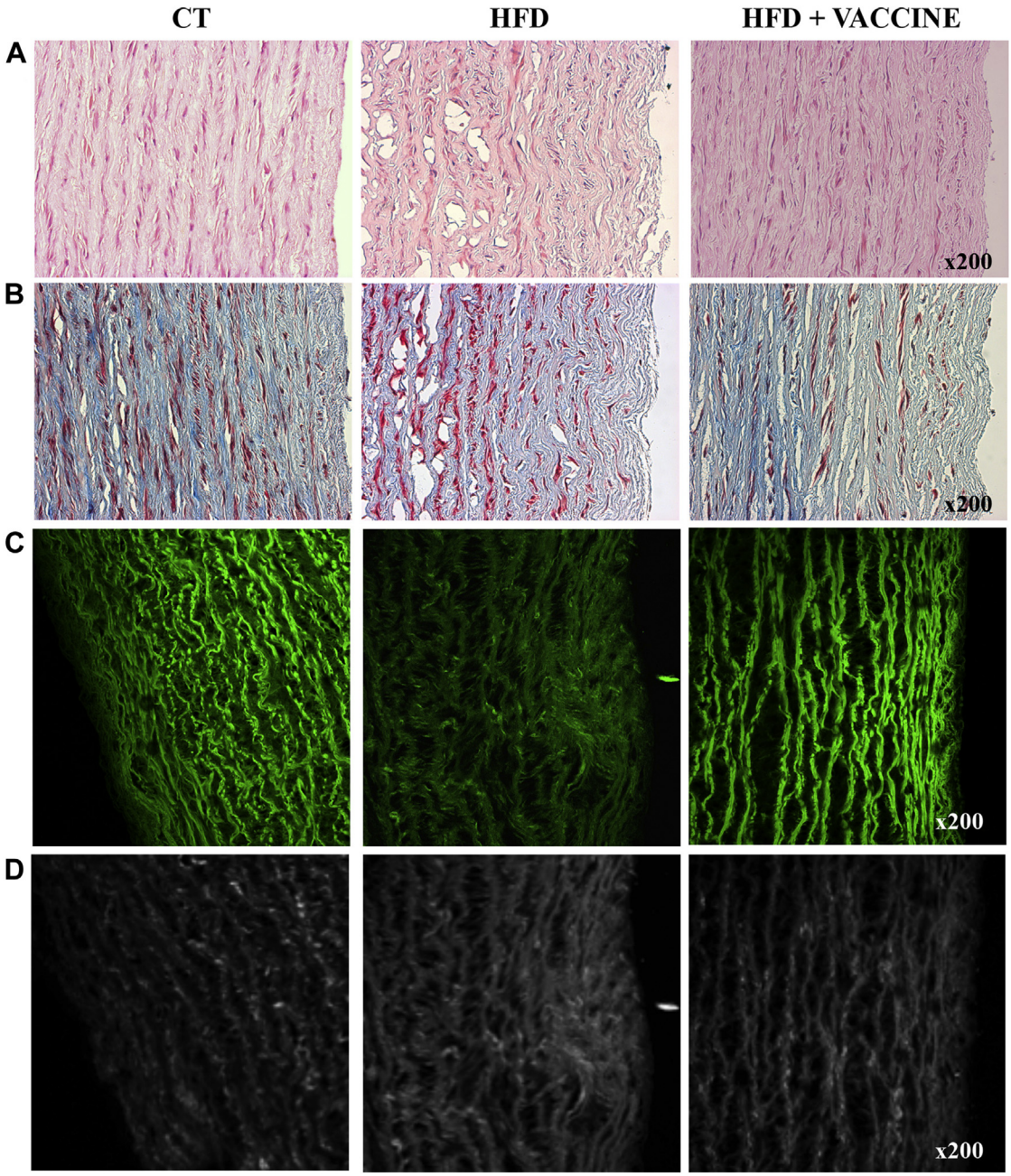

Figure 4. Vaccine HB-ATV-8 prevents the formation of atherosclerotic lesions induced by a HFD. Microscopic analysis of tissue sections from the thoracic aorta of a representative pig from each experimental group. Control group (CT), high fat diet group (HFD), and high fat diet + vaccine group (HFD + Vaccine). (A) Hematoxylin-eosin stain. (B) Masson's trichrome stain. (C) Two-photon excitation microscopy images. (D) Second-harmonic generation microscopy images.

lesions (Table 1). Moreover, collagen sheets observed in a widely distributed and spaced fashion, when studied with Masson's trichrome stain show a disordered and unevenly spaced arrangement. This can be clearly observed when the same samples are analyzed by two-photon excitation and second harmonic generation microscopy (Figure 4C and D). Using these imaging techniques, the connective fiber disorder found in aortas of pigs fed a HFD becomes evident denoting an important disarray among the different cell types that compose the vessel wall (Figure 4C and D). In contrast, the group of pigs that received the same HFD but was treated with vaccine HB-ATV-8 exhibit a decreased presence of lipid droplets and foam cells in the intima and media layers, and a smaller number of VSMC in the intima layer, similar to that shown in animals from the control group. More importantly, by two-photon excitation and second harmonic generation microscopy, a better ordered and a less spaced collagen fiber array together with a close to normal cellular architecture is observed (Figure 4C and D). Moreover, 50% of HB-ATV-8 treated pigs do not show any atherosclerotic lesions in their aortas and the remaining 50% only presented type I atherosclerotic lesions, in contrast to animals from the HFD group that showed type II or type III lesions (Table 1).

In order to obtain a better understanding at the molecular level of the histology changes observed with treatment, we analyzed the aortic expression of several genes known to be involved in vascular function such as the Krüppel-like factor-2 (*KLF2*), smooth muscle alpha-actin-2 (*ACTA2*), superoxide dismutase 1 (*SOD1*) and collagen type I alpha 1 (*COL1A1*) (Figure 5). KLF2 corresponds to an atheroprotective transcription factor that regulates expression of several vasoactive endothelial genes involved in the regulation of normal constriction/dilation of VSMCs (47), whereas SOD1 protects cells against cytotoxicity by scavenging superoxide radicals while being also involved in endothelial function by protecting nitric oxide release (48). *COL1A1* has been studied on the fact that corresponds to the most abundant protein associated to collagen fibrils (49). Furthermore, *ACTA2* has been employed as a cell marker for smooth muscle and associated to several occlussive diseases (50,51). So, consistent with tissue damage observed in our histology analysis associated to the HFD, there is a tendency for mRNA levels of these four genes to be upregulated in the aorta when compared to tissue from the control group (Figure 5). Interestingly, in all cases when gene expression is studied in the HFD group that received vaccine HB-ATV-8, the tendency is to go back to levels found associated to aortas from the control group (Figure 5).

We next assessed whether pigs fed a HFD present the typical hepatic alterations observed in NAFLD. Figure 6 shows sections of liver tissue from experimental animals of each group, displaying areas of hepatocytes around the central lobular vein. Hepatic tissue from control pigs presents the normal lobular histological characteristics. In contrast, hepatocytes from livers coming from pigs fed a HFD show microvesicular fat accumulation and signs of ballooning and inflammation. Interestingly, when the HFD + Vaccine group was examined, the frequency of these pathological features was considerably reduced (Table 2). It is important to mention that when compared to our previous findings employing a rabbit model (16), the current results show a less dramatic effect that might be related to the fact that the porcine model is considered refractory to develop not only fatty liver disease but also atherosclerosis in the same way as humans do (52). Nevertheless, when liver slides are studied by two-photon excitation and second harmonic generation microscopy, experimental animals from the HFD + Vaccine group, in comparison to the group that did not receive the vaccine, showed a decreased collagen deposition around the perisinusoidal and periportal areas and in general less fibrosis showing images close to the ones found in control tissue (Figure 6C and D).

Again, in order to initiate the exploration at the molecular of events that might be taking place with the administration of vaccine HB-ATV-8, we measured in liver tissue protein expression for the ATP-binding cassette transporter (ABCA1) defined as a cholesterol transporter that plays an important role in the homeostasis of cholesterol in the liver (53), and PPARα, reported to mediate fatty acid metabolism

Table 1. Effectiveness of vaccine HB-ATV-8 in decreasing aortic atherosclerotic lesions induced by a high fat diet measured at 4 and 7 months of treatment

	CT			HF			HF +VACCINE		
Lesion type	**4 months (n = 1)**	**7 months (n = 2)**	**Total (n = 3)**	**4 months (n = 4)**	**7 months (n = 2)**	**Total (n = 6)**	**4 months (n = 4)**	**7 months (n = 2)**	**Total (n = 6)**
TI	0	0	0	0	0	0	3 (75)	0	3 (50)
TII	0	0	0	3 (75)	1 (50)	4 (66.6)	0	0	0
TIII	0	0	0	1 (25)	0	1 (16.7)	0	0	0

CT, control group fed a standard diet; HFD, group fed a high fat diet; HFD + Vaccine, group fed a high fat diet + nasal administration of vaccine.
The histological classification of atherosclerotic lesions was carried out according to the American Heart Association guidelines (44).
Data in parenthesis represent percentage frequency within the specific number of experimental animals studied in each group.

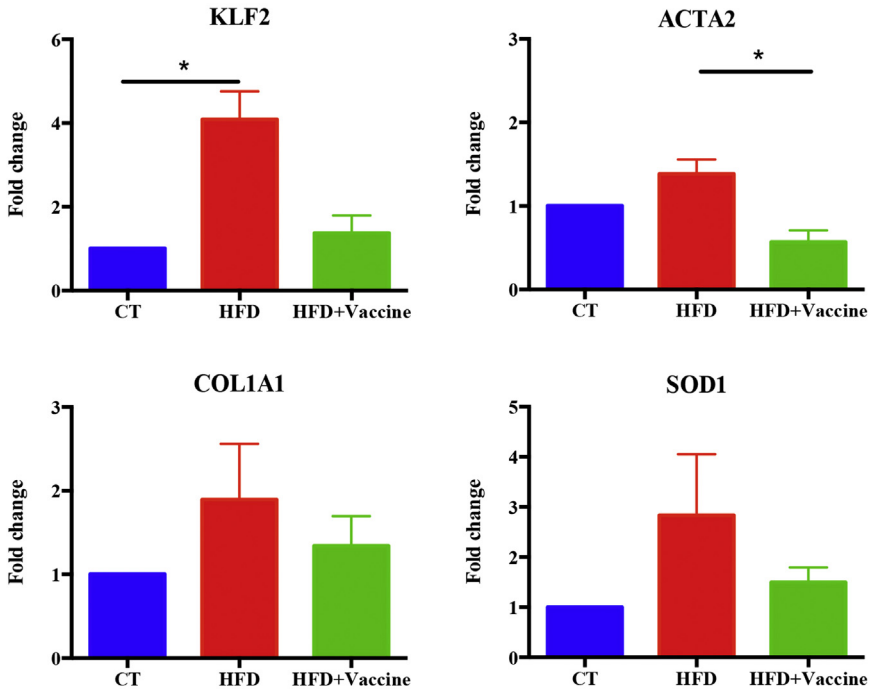

Figure 5. Vaccine HB-ATV-8 global effect on gene expression of *KLF2, ACTA2, COL1A1* and *SOD1* measured by qPCR in thoracic aorta samples. Control group (CT, $n = 3$), high fat diet group (HFD, $n = 6$), and high fat diet + vaccine treatment group (HFD + Vaccine, $n = 6$). Data presented as fold change with respect to actin expression. Data represented as mean ± S.E. $*p < 0.05$.

in the hepatocyte (54). Our analysis shows that although ABCA1 apparently is not significantly modified neither on animals fed with a HFD nor animals fed the HFD plus vaccine administration, there is a tendency for PPARα to increase in pigs fed a HFD that received the vaccine when compared to the HFD and control groups (Figure 7). These findings might support the possibility that a molecular compensatory effect at the cellular level might not only be carried out by the anti-CETP antibody but also by the peptide itself or one of the lipid components of vaccine HB-ATV-8.

Discussion

Since immunomodulation of key proteins that control the metabolism of cholesterol has been proposed as a prophylactic tool to treat atherosclerosis, vaccine HB-ATV-8 has been designed as a therapeutic strategy to reduce the development of atherosclerosis by generating autoantibodies against CETP. With support on our successful proof of concept carried out in the rabbit, vaccine HB-ATV-8 now has been tested in a porcine model. The pig as an experimental animal has gained interest in recent years since it has been shown that the evolutionary distance among many animal models used today, especially between rodents and humans, is a distant one (37). Preclinical studies performed in murine models can be useful in identifying new biochemical mechanisms or molecules and provide a platform for pharmacological development; nevertheless, as shown in the literature in many cases translation to a clinical setting has proven to be a difficult one. In contrast, the pig and the human share many characteristics, such as a similar anatomy and cardiovascular physiology, a situation that explains why cardiac output, mean arterial pressure and stroke volume are almost equivalent (55). Although the process of atherogenesis in pigs is difficult to develop given that many strains of farm pigs along time have been normally selected for an increase in protein deposition rather than fat accumulation (56), a HFD supplemented with cholesterol eventually promotes the development of artery lesions similar to those observed in the atherosclerotic disease in the human (5,57,58). Therefore, taking into account these considerations, the pig model can be considered an optimal model for the study of atherosclerosis and associated diseases such as NAFLD (59). Employing this experimental model and the use of a novel nanoparticle composition that serves as an immunogen when placed in the nasal mucosa, our study shows that modulation of the immune response continues to be a promising approach in the prevention of atherogenesis.

The present study shows that intranasal administration of vaccine HB-ATV-8 induces anti-CETP IgG antibodies, in turn reducing in parallel the presence of atherosclerotic lesions and hepatic damage in pigs fed a HFD. These results are also related to the fact that serum triglycerides remain at control values, in contrast to the not treated animal group fed a HFD. Even though triglycerides are not considered

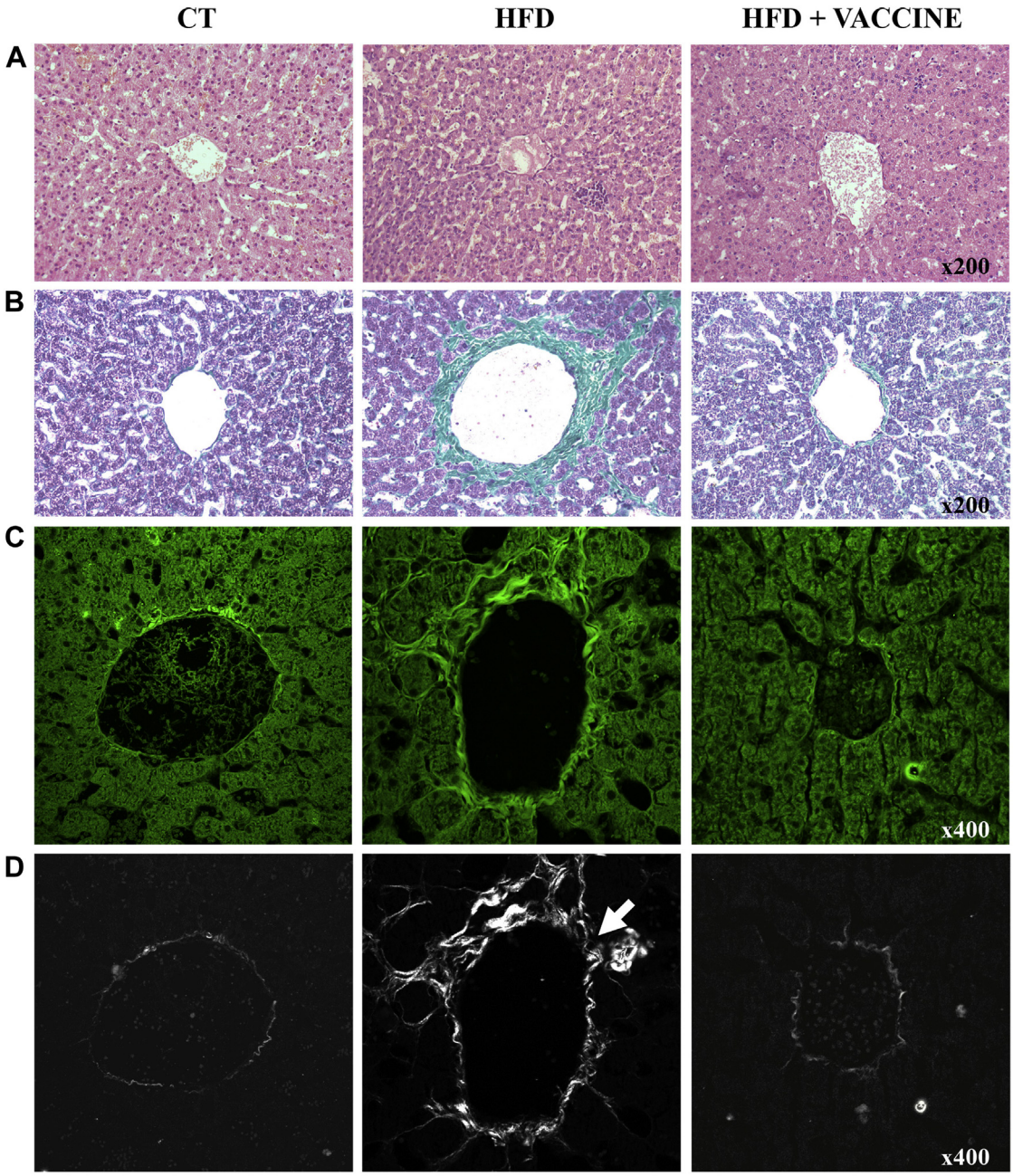

Figure 6. Vaccine HB-ATV-8 reduces hepatic inflammation and fibrosis induced by a HFD. Images show the central lobular vein from a liver sample of a representative pig from each experimental group. Control group (CT), high fat diet group (HFD), and high-fat diet + vaccine group (HFD + Vaccine). (A) Hematoxylin-eosin stain. (B) Masson's trichrome stain. (C) Two-photon excitation microscopy images. (D) Second-harmonic generation microscopy images. Arrow shows the presence of fibrosis.

atherogenic, it is well known that serum levels for this family of lipids are considered as a biomarker for CVD risk. This can be partially explained by their direct association with remnant lipoproteins and ApoCIII, a proinflammatory and proatherogenic apolipoprotein that inhibits the binding of ApoE and ApoB to proteoglycans and hepatic receptors, preventing clearance of these type of lipoproteins and therefore favoring their accumulation in the endothelium (60–63).

During an early stage of atherogenesis, cholesterol and other lipids penetrate the intima of arteries where their accumulation induce an inflammatory state and the activation of the immune system. As a consequence, macrophages infiltrate the tissue, start internalizing cholesterol and originate the formation of foam cells (44). Subsequently, foam cells are lysed, releasing lipids that further accumulate in the extracellular matrix. To decrease toxicity due to the extracellular deposition of lipids (64,65), VSMCs

Table 2. Vaccine HB-ATV-8 reduced hepatic inflammation and fibrosis induced by a high fat diet measured at 4 and 7 months of treatment

	CT			HF			HF + vaccine		
Histologic lesion	**4 months (n = 1)**	**7 months (n = 2)**	**Total (n = 3)**	**4 months (n = 4)**	**7 months (n = 2)**	**Total (n = 6)**	**4 months (n = 4)**	**7 months (n = 2)**	**Total (n = 6)**
Steatosis[a]	0	0	0	1 (25)	1 (50)	2 (33.3)	1 (25)	1 (50)	2 (33.3)
Ballooning	0	1	1 (33.3)	3 (75)	1 (50)	4 (66.7)	1 (25)	2 (50)	3 (50)
Inflammation	0	0	0	4 (100)	2 (100)	6 (100)	1 (25)	2 (100)	3 (50)
Fibrosis	0	0	0	4 (100)	2 (100)	6 (100)	2 (50)	1 (50)	3 (50)

CT, Control group fed a standard diet; HF, group fed a high-fat diet; HFD + Vaccine, group fed a high fat diet + nasal administration of vaccine.
Data represent number of cases by each category and data in parenthesis percentage frequencies.
Fibrosis reported in these samples was mainly portal or perisinusoidal and portal/periportal fibrosis.
[a]Microvesicular steatosis.

migrate and begin internalizing excess lipid in support of macrophage function. Although VSMCs are responsible for remodeling the arterial wall, a prolonged state of oxidative stress causes their trans-differentiation to macrophage-type cells (66). These cells are able to phagocytize and present antigens, favoring the inflammatory state (67,68). Moreover, the structure of the arterial wall specifically depends on a delicate equilibrium between the synthesis and degradation of extracellular matrix proteins such as collagen and elastin, where an acute change in this equilibrium might play an important role during the process of atherogenesis. In this sense, an uncontrolled degradation of proteins of the extracellular matrix carried out by proteases such as metalloproteinase-1 and -9, might induce further vascular damage. This in turn promotes atherogenesis through the trans-endothelial migration of leukocytes, migration and proliferation of VSMCs, neovascularization, vascular cell apoptosis, and finally the formation of a neointima that might progress until the rupture of the aortic wall takes place (69). Together with cells of the immune system involved in atherosclerosis such as monocytes converted into macrophages, new studies have pointed to mast cells and stellar cells as critical cell types important in collagen degradation and smooth muscle survival (70). Mast cells produce and release matrix metalloproteases together with proinflammatory molecules such as interferon and interleukin-6 inhibiting the proliferation of smooth muscle cells and also promoting the process of apoptosis (70–73).

Since the use of two-photon excitation and second harmonic generation microscopy has been extremely useful to study the extracellular matrix and to visualize collagen fibers in association to a classical light microscopy, the study of viable arteries *ex vivo* gives us a better understanding of the functionality and dynamic behavior of the artery wall (74,75). During the course of our study, we observed that vaccinated animals fed a HFD did not show the clear disarray of extracellular matrix observed in the aortas of

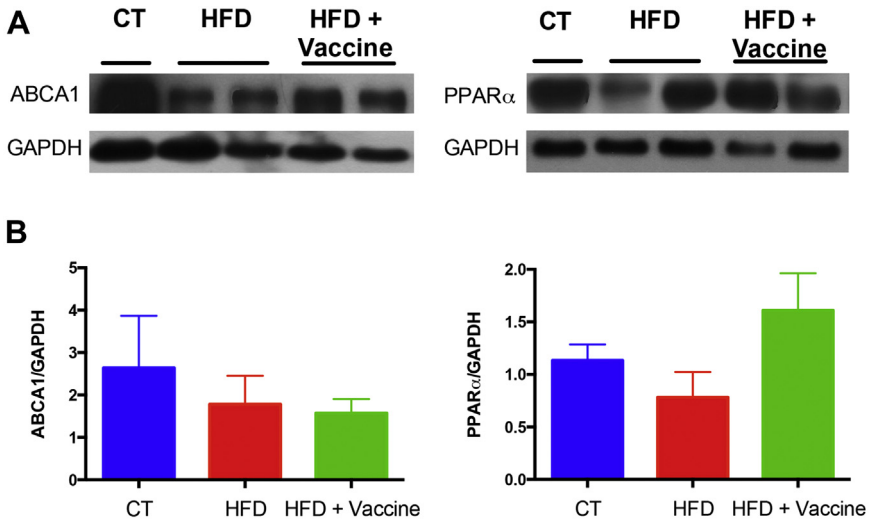

Figure 7. Vaccine HB-ATV-8 effect on hepatic PPARα and ABCA1 concentration. (A) Western blot analysis of samples obtained from one representative control animal and two representative samples from the HFD and HFD + vaccine groups. (B) Semicuantitative protein correlation between expression of ABCA1 and PPARα with GAPDH. Control group (CT, n = 3), high fat diet group (HFD, n = 6), and high fat diet + vaccine treatment group (HFD + Vaccine, n = 6). Data represented as mean ± S.E.

animals that were only fed a HFD without vaccination. This phenomenon is shown to be accompanied by an increased expression of *KLF2, ACTA2, COL1A1 and SOD1* mRNA's in the aortas of animals fed exclusively the HFD. Interestingly, this increase in mRNA levels is reversed close to control values in animals fed a normal diet when the HFD plus vaccination group is studied, suggesting that vaccine HV-ATV-8 might be also considered as a modulator of anti-atherosclerotic signals. It is well known that a HFD induces vascular dysfunction upregulating pathways associated to the production of reactive oxygen species (ROS), oxidative stress, and release of proinflammatory adipokynes/cytokynes (76–78). KLF2 inhibits both the expression of inflammatory cytokynes and the production of adhesion molecules such as VCAM-1 and E-selectin, known to be critical for leukocytes recruitment and extravasation (79,80). In addition, aortas from pigs fed a HFD show a disordered pattern of collagen fibrils, that to a certain extent, is less apparent in aortas isolated from animals fed the HFD plus vaccination, also in agreement with results showing a tendency to present a lower mRNA concentration and synthesis of type I collagen. Interestingly, during the development of an atherosclerotic plaque, the extracellular matrix composition of a vessel undergoes pronounced changes, where VSMCs initially showing a contractile phenotype are transformed to present a synthetic phenotype with the consequent increase in the deposit of types I and III collagen, elastin and fibronectin (49).

On the other hand, steatohepatitis and atherosclerosis share several characteristics such as lipid accumulation and the development of an inflammatory state (45,81). During the present study, pigs fed a HFD in addition to the presence of atherosclerotic changes, also developed hepatic disease characterized by microvesicular steatosis, ballooning, cellular inflammation, and fibrosis. Two-photon and second harmonic generation imaging clearly show the enormous difference in collagen deposition observed in liver tissue obtained from the group of animals exclusively fed the HFD and those treated simultaneously with vaccine HB-ATV-8. In general, experimental animals fed the HFD independently if they received or not the vaccine, do not show macrovesicular steatosis. This phenomenon is most probably due to the relatively short period of time used to carry out the study before animal sacrifice (56). Nevertheless, the presence of inflammation and fibrosis was less evident in the HFD + Vaccine group in comparison to HFD fed pigs.

The tendency for PPARα to increase in liver cells of animals fed a HFD that received the vaccine supports an explanation for the reduced level of plasma triglycerides found in this group of animals. PPARα considered a transcription factor that regulates the expression of genes involved in VLDL production, lipid trafficking and triglyceride-rich lipoprotein clearance, apparently helps suppress the acute-phase response and inflammation in the liver (82–84) and in conjunction with PPARβ/δ, shown to improve steatosis, inflammation and fibrosis in preclinical models of NAFLD (83,85).

Since our findings show that vaccine HB-ATV-8 decreases fat accumulation and most probably an inflammatory state in the artery wall and liver, it is important to point out that even though an auto-anti-CETP antibody titer was clearly detected around 4 months of treatment, at this time the effect was already observed. We also hypothesize that in addition to the regulation of lipid transport carried out by CETP due to the effect of the auto-anti-CETP antibody, there might be additional protective mechanisms that could be also related to a direct effect given by one of the components of the vaccine preparation, such as the peptide itself. This possibility is supported by studies of a series of peptides that promote the efflux of cholesterol from cells (86). Our group has also shown using cultured macrophages, a decrease in foam cell formation and down-regulation of CD36 and ACAT-1 when the peptide itself is tested (87,88). Evidence also shows that several other peptides interacting with endothelial cells, VSMCs, monocytes and/or macrophages, might be used for therapeutic purposes in the control of atherosclerosis (89–91). Since the HFD group independently if vaccinated or not maintain the same gain in body weight along the study, we speculate that vaccine HB-ATV-8 might also promote the accumulation of lipids in other tissues such as the adipose tissue. Current experiments are being carried out to establish this possibility.

Among the series of drugs offering to decrease the risk of cardiovascular disease by increasing the serum concentration of HDL-C, several CETP inhibitors obtained by chemical synthesis have been studied (92). Several other strategies include the use of fibrates (93) and antioxidants (94), but at the end also apparently ineffective to decrease the risk of CVD. Considering CETP as a plasma protein that transfers cholesteryl-esters among lipoproteins, specifically HDL to VLDL and LDL, it has been described as a molecule that promotes atherogenesis; therefore, the potential inhibition of its activity by new molecules has attracted attention in an effort to increase HDL-C and decrease coronary artery disease (95). Nevertheless, the use of these chemically synthesized compounds in the strategy to inhibit CETP has shown to be troublesome (92,95,96). Torcetrapib was the first CETP inhibitor to be tested in Phase 3 clinical trials, but had to be prematurely terminated due to a series of cardiovascular problems and mortality associated with non-cardiovascular events (97–100) such as infection and off-target aldosteronism as presented in the ILLUMINATE, RADIANCE and ILUSTRATE trials (97,100,101). These studies showed an increase in HDL-C and a decrease in LDL-C but no impact in the presence of atheroma lesions (100,102).

During the Phase 2 clinical trial of CETP inhibitor dalcetrapib, the study had to be stopped due to off-target side

effects and ineffectiveness shown by a non-significant effect on plasma LDL-C levels despite a moderate increase in HDL-C (103). Also, the OUTCOMES phase 3 trial had to be halted due to a lack of clinically meaningful efficacy (104). Although evacetrapib also improved lipoprotein biomarker distribution, treatment did not result in a lower rate of cardiovascular events among patients with high-risk vascular disease (105). So far, anacetrapib seems to show a better effect on various coronary events studying patients presenting atherosclerotic vascular disease but only under intensive statin therapy (106). Taken these data together, due to the presence of side effects and off-target activity, there seems that chemically synthesized CETP inhibitors need more time to be completely understood.

The example of torcetrapib could be considered a prototypical one since we now know that CETP binding sites for this molecule are shared with proteins of the Plunc family including an isoform of CETP named by our group as CETPI and identified as a lipopolysaccharide binding protein (107). According to our results, an non-specific binding phenomenon might have been directly related to sepsis and the mortality rate shown in the ILLUMINATE trial (108−110) since torcetrapib might have had an unknown secondary effect disabling the main function of CETPI directly related with the inactivation of LPS in plasma.

In contrast to the several strategies discussed in this study used to inhibit the function of CETP, the induction of an immune response by self-generating anti-CETP antibodies shows many advantages above other approaches to treat the process of atherogenesis (20,111−113). The present investigation studying a porcine model corroborates our previous reports showing that intranasal therapeutic vaccine HB-ATV-8 is effective in the control of the process of atherogenesis and the development of fatty liver disease, associated to important advantages such as simplicity of design and application combined to a low production cost.

Conclusion

Considering that for many years no new synthesis of lipid metabolism modulator molecules have been successfully introduced into clinical practice, the immunological approach using the generation of autoantibodies against key proteins involved in the physiopathology of atherosclerosis have come into play. Among the several immunological approaches used to inhibit CETP function, vaccine HB-ATV-8 seems to be the only one that offers non-invasive intranasal vaccination employing a micellar nanoparticle preparation composed of a mixture of common lipids and a peptide together with the use a simple delivery device. Since vaccination ameliorates atherosclerotic and hepatic lesions caused by a HFD improving triglyceride metabolism and promoting atheroprotective and anti-inflamatory signals, vaccine HB-ATV-8 offers a brand new approach to prevent the process of atherogenesis and associated NAFLD in the human.

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Supplementary Data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.arcmed.2019.01.007.

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