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CHARACTERISATION OF ATHEROGENIC EFFECTS OF LOW CARBOHYDRATE, HIGH PROTEIN DIET (LCHP) IN APOE/LDLR$^{-/-}$ MICE

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Abstract: *Introduction:* Low Carbohydrate High Protein diet represents a popular strategy to achieve weight loss. *Objective:* The aim of this study was to characterize effects of low carbohydrate, high protein diet (LCHP) on atherosclerotic plaque development in brachiocephalic artery (BCA) in apoE/LDLR$^{-/-}$ mice and to elucidate mechanisms of proatherogenic effects of LCHP diet. *Materials and Methods:* Atherosclerosis plaques in brachiocephalic artery (BCA) as well as in aortic roots, lipoprotein profile, inflammation biomarkers, expression of SREBP-1 in the liver as well as mortality were analyzed in Control diet (AIN-93G) or LCHP (Low Carbohydrate High Protein) diet fed mice. *Results:* Area of atherosclerotic plaques in aortic roots or BCA from LCHP diet fed mice was substantially increased as compared to mice fed control diet and was characterized by increased lipids and cholesterol contents (ORO staining, FT-IR analysis), increased macrophage infiltration (MOMA-2) and activity of MMPs (zymography). Pro-atherogenic phenotype of LCHP fed apoE/LDLR$^{-/-}$ mice was associated with increased plasma total cholesterol concentration, and in LDL and VLDL fractions, increased TG contents in VLDL, and a modest increase in plasma urea. LCHP diet increased SCD-1 index, activated SREBP-1 transcription factor in the liver and triggered acute phase response as evidence by an increased plasma concentration of haptoglobin, CRP or AGP. Finally, in long-term experiment survival of apoE/LDLR$^{-/-}$ mice fed LCHP diet was substantially reduced as compared to their counterparts fed control diet suggesting overall detrimental effects of LCHP diet on health. *Conclusions:* The pro-atherogenic effect of LCHP diet in apoE/LDLR$^{-/-}$ mice is associated with profound increase in LDL and VLDL cholesterol, VLDL triglicerides, liver SREBP-1 upregulation, and systemic inflammation.

Key words: LCHP diet, apoE/LDLR$^{-/-}$ mice, BCA, development of unstable lesions.

Introduction

Dietary factors play a crucial role in the development of atherosclerosis. High fat, high calorie diets are known risk factors for this disease. Additionally, there is strong evidence that dietary animal proteins also can contribute to the development of atherosclerosis (1, 2).

A low-carbohydrate diet, characterized by William Banting in the 1860s, has currently received much attention due to Dr. Atkins' diet. Rapid initial weight loss, which can be psychologically encouraging, may be the result of this diuretic effect (3). In general, although LCHP diet may improve some metabolic markers, particularly in type 2 diabetes mellitus (T2D) or the metabolic syndrome (MS), their net effect on arterial wall function seems detrimental. Indeed, low or very low carbohydrate content may lead to impaired small artery vascular function (4). Moreover, in a cohort of patients at increased cardiovascular (CV) risk the diet characterised by a low amount of carbohydrate, but high amounts of protein and fat, was associated with a poorer small artery vascular reactivity (5).

Recently, a study by Foo et al. (6), provided the first evidence, that atherosclerotic mice (apoE$^{-/-}$) placed on a 12-week low carbohydrate high protein diet (LCHP) showed a significant acceleration of atherosclerosis development. Authors suggested that this phenomenon was not due to alterations in serum cholesterol, NEFA, inflammatory mediators or infiltrates, oxidative stress but due to substantially reduced number of bone marrow and peripheral blood endothelial progenitor cells (EPCs), and impaired vascular regenerative capacity. EPCs from mice on a LCHP diet manifested lower levels of activated (phosphorylated) Akt, a serine-threonine kinase important in EPC mobilization, proliferation and survival resulting in reduced ischemia-induced neovascularization in LCHP diet fed apoE$^{-/-}$ mice. Although impairment of EPC function in response to LCHP diet represent an interesting and novel finding, it is unlikely to fully explain the atherogenic effects of LCHP diet.

In our previous work, using another model of genetically modified hyperlidemic mice such as apoE/LDLR$^{-/-}$ mice

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(7) we confirmed that the LCHP diet significantly increased the extent of advanced atherosclerosis in the aortic root and may promote plaque vulnerability. We also demonstrated that LCHP-fed apoE/LDLR$^{-/-}$ mice developed more extensive atherosclerosis than WD-fed mice, despite similar dietary fat and cholesterol contents but in our previous work we did not provide insight into mechanisms of pro-atherogenic effects of LCHP diet.

Accordingly, the aim of this study was to evaluate the effect of low carbohydrate, high protein diet on apoE/LDLR$^{-/-}$ mice in more details, with the aim to elucidate possible mechanisms of pro-atherogenic action of LCHP diet.

Atherosclerosis plaques in brachiocephalic artery (BCA) were comprehensively analyzed in Control (AIN-93G) or LCHP (Low Carbohydrate High Protein) diet fed mice. In addition, effects of LCHP on lipoprotein profile using novel technique of flow field fractionation (FFF), on fatty acids contents in the liver (GC/MS), cholesterol contents in atherosclerotic plaques (FT-IR) as well as inflammation biomarkers, expression of SREBP-1 in the liver were studied. In order to assess the significance of pro-atherogenic effect of LCHP diet, long term survival in LCHP (Low Carbohydrate High Protein) diet fed mice as compared to control diet fed mice was analyzed.

Materials and methods

Animals and housing

ApoE/LDLR$^{-/-}$ male (for survival experiment) and female (for characterization of atherosclerotic plaque) mice (8) were originally obtained from Jackson Lab (USA) and bred in house. ApoE/LDLR$^{-/-}$ mice at the age of 16-18 weeks with pre-established atherosclerosis were used in this study. Animals were housed in colony cages in a temperature-controlled environment (22-25°C) with a 12 hour light/dark cycle. They had free access to food and water.

All procedures involving animals were conducted according to the Guidelines for Animal Care and Treatment of the European Union and were approved by the local Animal Ethics Commission.

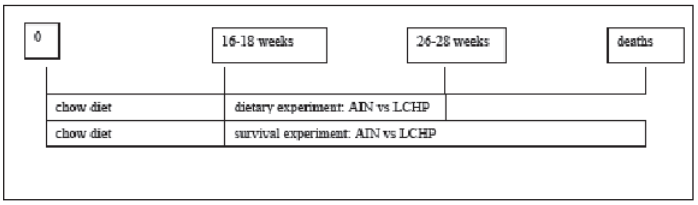
Diets and feeding

For the survival experiment up to the age of 16-18 weeks, the mice were fed a commercial, cholesterol-free, pelleted diet (Sniff M-Z Spezialdiäten GmbH; Soest, Germany). At the age of 16-18 weeks mice were weight-matched between groups (n=40 in each group). The experimental diets were: Control - AIN-93G diet, according to Reeves, et al (9) (protein: 200g/kg diet, corn starch: 532g/kg diet, fat: 70g/kg diet, energy: 3558kcal/kg diet) and LCHP (protein: 524g/kg diet, corn starch: 50g/kg diet, fat: 210g/kg diet, energy: 4136kcal/kg diet). All deaths were recorded (Figure 1).

For characterization of atherosclerotic plaque and understanding the mechanism of LCHP action, the mice were fed a commercial, cholesterol-free, pelleted diet up to the age of 16-18 weeks. At the age of 16-18 weeks mice were weight-matched between groups (n=12 in each group) and fed AIN-93G-based diets: control diet (AIN) (9) and experimental LCHP diet for the next 10 weeks (Figure 1). Body weight of mice were monitored weekly.

Figure 1

Experimental design



After 10 weeks of feeding, the mice were injected intraperitoneally with 1000 IU of heparin (Sanofi-Synthelabo; Paris, France) and after 10 min, anesthetized with 40 mg/kg of sodium thiopental (Biochemie; Vienna, Austria) given intraperitoneally and finally sacrificed by cervical dislocation.

Blood sampling and measurements of biochemical markers

Blood samples were taken from the vena cava and were collected into test tubes and centrifuged (4 000 x g, 10 min) to obtain plasma samples. The samples were deep frozen (-80°C) and stored until further analysis using commercially available kits for alanine transaminase (ALT), aspartate aminotransferase (AST), lactate dehydrogenase (LDH), urea, protein and creatinine (Pentra 400, Horiba). Multiplexing technique using magnetic beads in a Bio-Plex 200 system (MILLIPLEX MAP Kit Mouse Acute Phase Magnetic Bead Panel, No MAP2MAG-76K, EMD Millipore Corporation, USA) was performed for measurement of acute phase response (Haptoglobin, Serum Amyloid Protein/Pentraxin-2 – SAP, C-Reactive Protein – CRP Alpha-1 Acid Glycoprotein - AGP, Alpha-2 Macroglobulin - A2M).

Determination of cholesterol and triglyceride concentration as a function of lipoprotein size

Field Flow Fractionation (FFF) has been used before to separate lipoproteins according to size and determine the cholesterol and triglyceride concentration for each size fraction (10). Baseline separation of HDL and LDL can be achieved as well as separation of VLDL species up to more than 100 nm size. Cholesterol and triglyceride concentration of eluting fractions is determined with post-separation enzymatic reaction and UV detection at 500 nm which, based on calibration, produces absolute concentration in mg/dl. In addition the size of eluting fractions can be calculated using FFF theory. For the first time in a clinical study, Hollow-Fiber-Flow-FFF (HF5) (11) has been applied, which has the advantage to allow full quantitative characterization of lipoprotein profiles using only 1μl of serum sample. The HF5 system used is

fully automated which made it possible to process the serum of every individual mouse, a critical requirement in order to compare the lipoprotein profiles of the two mouse groups and assess the statistical significance of differences. The FFF system in use was a Wyatt Eclipse DUALTEC (Wyatt Technology Europe, Dernbach, Germany) in combination with an Agilent HPLC system (Agilent Technologies, Santa Clara, CA, USA), consisting of an online degasser, an isocratic pump, an autosampler and a variable wavelength detector (all out of 1200 series). Furthermore, a Shimadzu CTO-20 AC column oven (Shimadzu, Kyoto, Japan) was used for thermostatization of the enzymatic reaction at 310 K and a Shimadzu LC-20 AD HPLC pump (Shimadzu, Kyoto, Japan) for delivering the enzyme buffer (Biolabo, Maizy, France). The post-separation colorimetric reaction was performed by applying the CHOD-PAP method using a knitted reaction coil, which consisted of 0.5 mm ID PTFE tubing with a length of 15 m, thus creating a reaction volume of approximately 3 cm^3 and resulting in a total reaction time of 5 minutes (total reaction flow was 0.6 mL min^{-1}). The hollow fiber membrane (Microdyn-Nadir, Wiesbaden, Germany) with an inner diameter of 0.8 mm was made of polyethersulfone and had a nominal molecular weight cut-off of 10 kDa. Hydrodynamic size has been calculated using the software ISIS V.1.2 (Wyatt Technology Europe GmbH).

Determination of fatty acids composition in liver

The samples (20 mg) were placed in vials and treated with 2 ml solution of 0.5 M KOH in methanol. The samples were heated at 60°C for 10min. Next, 2 ml of 14% BF_3 in methanol was added and heated at 60°C for 10min. After cooling, 2 ml of hexane and 2 ml saturated sodium chloride was added. The mixture was vortexed. The upper n-hexane layer was transferred to eppendorf tubes and dried with anhydrous Na_2S0_4. The analysis of FAME was performed on a SHIMADZU GC-MS- QP 5050A equipped with a SP-2560 capillary column (100m x 0.25mm i.d. x 0.25μm film thickness, Supelco). Helium was the carrier gas and operated at flow rate of 1.8 ml/min. Injector temperature was maintained at 245ºC, detector temperature was 200ºC. The total FAME profile in a 1μl injection at a split mode was determined. The oven temperature was operated at 60°C for 5min, then the temperature programmed at 5°C/min to 180°C, held for 16min, programmed at 5°C/min to 220°C, held for 7min. FAME identification was validated and based on electron impact ionization mode.

Quantitation of atherosclerosis in aortic roots and brachiocephalic arteries (BCA)

The heart and ascending aorta were dissected and then embedded in OCT compound (CellPath, Oxford, UK) and snap-frozen. 10μm serial cryosections of the aortic root were cut using a standardized protocol. After fixation in 4% buffered formalin (pH=7), sections were stained with oil-red-O and Sirius Red (Sigma-Aldrich, St. Louis, MO, USA) and examined under an Olympus BX50 (Olympus, Tokyo, Japan) microscope. Additionally, sections were stained by zymography. Images of the aorta were recorded using a digital camera and stored as TIFF files of resolution 2048×1536 pixels or 1024x768 pixels. The total area of the lesion was measured semi-automatically in each slide using LSM Image Browser 3.2 Software (Zeiss, Jena, Germany). For each animal, mean lesion area was calculated from nine sections.

Brachiocephalic arteries were dissected, fixed in 4% buffered paraformaldehyde (pH 7.4) and embedded in paraffin. Sections were cut every 10μm along the artery (starting from the proximal end) and stained with hematoxylin and eosin and OMSB. Area of the plaque, lumen, lipid core was calculated. Sections stained resorcin-fuchsin method for elastin were inspected for the presence or absence of cap disruption and buried fibrous layers in the plaque.

Macrophages immunohistochemistry staining

For the detection of macrophages slides were incubated with anti-avidin and anti-biotin solutions (Vector Laboratories, USA). Thereafter, slides were incubated with primary antibodies MOMA2 for 1 h at room temperature and were developed with biotin-conjugated goat anti-rat Ig (dilution 1:400 in bovine serum albumin) (Vector Laboratories) in the presence of 200μg/ml normal mouse IgG (Dako, Denmark) afterwards. Antibody reactivity was detected using horseradish peroxidase-conjugated biotin – avidin complexes (Vector Laboratories) and developed with diaminobenzidine tetrahydrochloride substrate (Dako). Photographic documentation and image digitizing from the microscope were performed with the Olympus AX 70 and a digital firewire camera Pixelink PL-A642 (Vitana Corp, Ottawa, Canada) with image analysis software NIS (Laboratory Imaging, Czech Republic). Stereological analysis was performed with a PointGrid module of the ELLIPSE software (ViDiTo, Kosice, Slovakia).

Cholesterol and proteins quantification by FT-IR measurements

FT-IR imaging experiments were performed using the Varian 620-IR microscope coupled to a 670-IR spectrometer with a liquid nitrogen cooled mercury-cadmium-telluride (MCT) and 128x128 pixel focal plane array (FPA) detector. Measurements were obtained in transmission mode. For background and sample measurements 128 and 32 scans were collected. The total acquisition time was approximately 5-10 min for each image. All spectra were obtained with the spectral resolution of $4cm^{-1}$. Data managing and visualization was done in MatLab 10 environment (The MathWorks, Natick, MA) with in-home built scripts.

For each of the sections (4-8 sections per animal), a median of the respective integrations of cholesterol band (1040-1070cm^{-1}) and amide I band for proteins (1700-1600cm^{-1}) was taken as a measure of chemical composition.

Real-time qRT-PCR

Total RNA from liver sample was isolated by using a "Total RNA" kit (Qiagen, Germany) according to the manufacturer's protocol. Isolated RNA samples were dissolved in RNase-free water, and RNA quality, integrity and quantity was measured with the use of NanoDrop (NanoDrop Technologies, USA).

Expression of the selected genes (acyl-CoA oxidase - ACO; fatty acid synthase FAS; sterol regulatory element binding proteins - SREBP;) was checked by qRT-PCR. A TaqMan gene expression assay kits were used. The housekeeping gene β-actin was used for normalisation. Results are presented as the ratio of genes to β-actin expression (Table 1C).

Table 1A

Quantitative results of TG (mg/dL) and cholesterol (mg/dL) in mice's lipoprotein fed: Control - AIN-93G; LCHP (Low Carbohydrate High Protein) diets. Data are means ± SEM. The means bearing different letters were significantly different (p<0.05).

	AIN	LCHP
Cholesterol (sum)	1219.4±49.2^a	2244.4±127.7^b
Quantitative results of cholesterol		
- HDL	27.2±2.3^a	15.2±1.9^b
- LDL	247.5±32.0^a	522.7±44.8^b
- VLDL	944.7±60.4^a	1706.5±89.3^b
LDL/HDL	9.3±1.2^a	38.1±9.9^b
(VLDL+LDL)/HDL	45.5±4.9^a	161.1±37.3^b
Quantitative results of TG		
- VLDL	32.6±6.7ns	66.6±16.7ns

Statistical analysis

Results are expressed as mean ± SEM. Where appropriate, the data were subjected to analysis of variance calculated in the STATISTICA 10 package (StatSoft Inc., USA), followed by post-hoc Duncan's multiple range test (means were considered significant at P<0.05). The data resulting from quantification of atherosclerosis were analyzed by the non-parametric Mann-Whitney test. Differences were considered significant at P<0.05.

Results

Effects of LCHP diet on the development of atherosclerosis

The areas of plaques as measured in the aortic root were significantly increased in the LCHP group (Figure 2A). MMP significantly increased in mice fed LCHP diet (Figure 2B). It was shown that the plaque area in BCA also tended to increase in LCHP vs control group (253078 vs 293576 μm^2) (Figure 3A, 3B, 3C, 3D). Lumen area tended to decrease after LCHP feeding (69993,76 vs 48425,36μm^2). Moreover, lipid core was significantly increased in LCHP group (at 400 and 525 μm) compared to mice fed control diet. Macrophages area in BCA significantly increased in LCHP group (Figure 3E). Additionally, the number of buried fibrous caps was increased in LCHP group as compared to control group (2.3 vs 1.9).

Figure 2

Area of atherosclerosis (A), area of MMP (B) and representative images of cross-sections of aortic roots showing aortic plaque stained ORO and MMP in mice fed: Control - AIN-93G; LCHP (Low Carbohydrate High Protein) diets. The means bearing different letters were significantly different (P< 0.05)

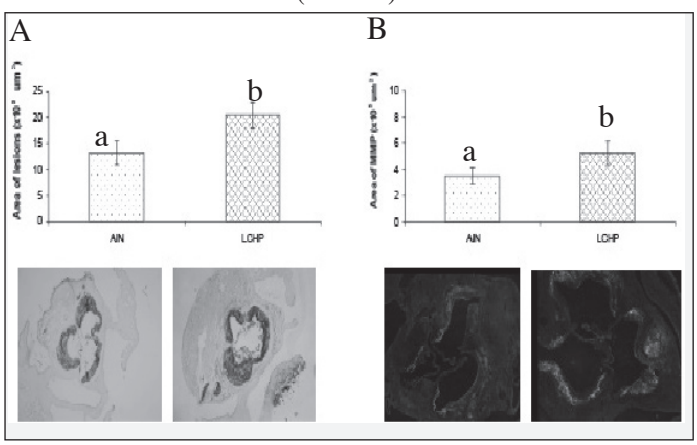


Table 1B

Fatty acid composition in liver, wt. %

	AIN	LCHP
12:0 dodecanoic acid	0±0	0,66±0.11
14:0 tetradecanoic acid	0,87±0,08^a	5,28±0,50^b
14:1 tetradecenoic acid isomer	0±0	0,58±0,07
15:0 pentadecanoic acid	0,1±0^a	0,81± 0,05^b
16:0 hexadecanoic (palmitic) acid	18,75± 0.95^a	24,98±1,09^b
16:1 hexadecenoic acid isomer	0,34±0,04^a	0,71± 0,04^b
16:1 hexadecenoic (palmitoleic) acid	3,35±0,35^a	8,44±0,73^b
17:0 heptadecanoic acid	0,14± 0,02^a	0,39±0,02^b
17:1 heptadecenoic acid isomer	0±0	0,54±0,05
18:0 octadecanoic (stearic) acid	3,41± 0.,58^a	2,71±0,15^b
18:1 octadecenoic (oleic) acid	37,52± 1,58^a	49,58±2,08^b
18:2 9,12 octadecadienoic (linoleic) acid	33,47± 1,76^a	4,23±2.09^b
18:3 9,12,15 octadecatrienoic acid	2,15± 0,37^a	1,09± 0,13^b
SFA	23,17±1,48^a	34,83±1,78^b
MUFA	41,20±1,78^a	59,85±2,09^b
PUFA	35,62 ±1.94^a	5,33±1,98^b
16:0/18:2 n-6	0,56± 0,02^a	7,01± 0,03^b
16:1n–7/16:0	0,18 ±1.87^a	0,34± 1,31^b
18:1/18:0	11,24 ±0,72^a	18,38±0,49 ^{b}

Figure 3A
Representative pictures of brachiocephalic arteries (BCA) in mice fed: Control - AIN-93G and LCHP (Low Carbohydrate High Protein) diets stained with OMSB

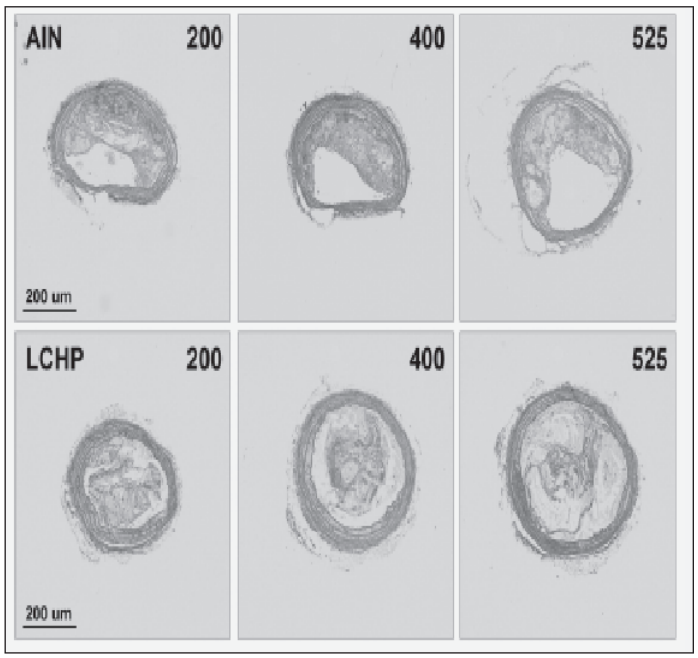


Table 1C
Genes expression in mice's liver fed: Control - AIN-93G; LCHP (Low Carbohydrate High Protein) diets

	AIN	LCHP
FAS [a.u.]	1	2.33
SREBP [a.u.]	1	1.44
ACO [a.u.]	1	0.56

SREBP, sterol regulatory element binding proteins; ACO, acyl-CoA oxidase; FAS, fatty acid synthase in apoE/LDLR$^{-/-}$ mice fed experimental diets. The target genes were normalized to β-actin and expressed in arbitrary units [a.u.].

Cholesterol and proteins quantification by FT-IR measurements shown differences of cholesterol and proteins in BCA sections of apoE/LDLR$^{-/-}$ mice fed LCHP diet versus a control diet. The means of LCHP and AIN groups along with standard deviation were shown in Figure 3F, and 3G. A significant increase in cholesterol content and a decrease in protein content were observed.

Effects of LCHP diet on plasma lipid profiles

The effects of LCHP diet on plasma lipid profile are shown in Table 1A. The high resolution analysis using HF5 reveals significant and dramatic changes not only in the absolute concentration, but even more in the distribution of cholesterol as a function of lipoprotein size. The increasing total cholesterol concentration of fasting mice fed LCHP diet was due mostly to the increase in the LDL and VLDL fractions. The LCHP diet increased cholesterol in LDL and VLDL approximately by 2-fold as compared to control diet. Cholesterol level in HDL fraction was significantly decreased in LCHP groups as compared to AIN diet. Content of TG in VLDL was also increased in LCHP fed mice but the difference did not reach statistical significance.

Figure 3B
Area of plaque (%) in mice fed: Control - AIN-93G and LCHP (Low Carbohydrate High Protein) diets.

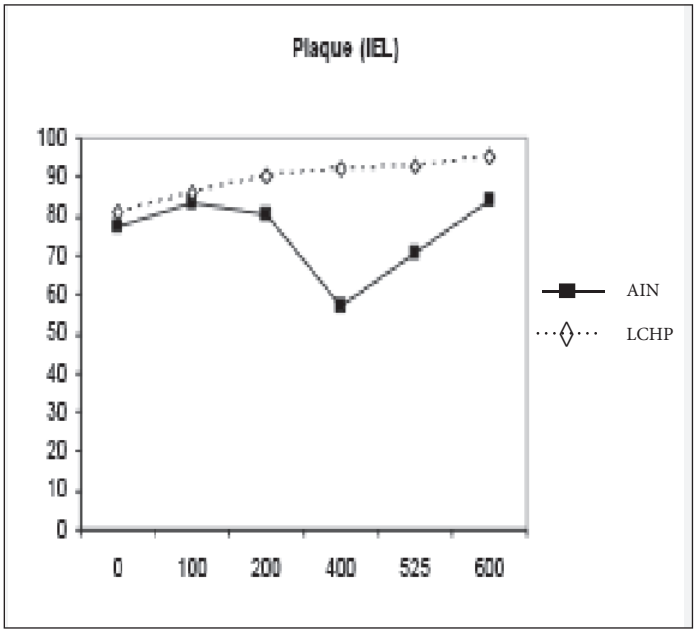


Table 2
Acute phase proteins levels in mice's plasma fed: Control - AIN-93G; LCHP (Low Carbohydrate High Protein) diets. The means bearing different letters were significantly different ($p<0.05$)

	AIN	LCHP
Haptoglobin [ug/ml]	84,82±68,79 ns	205,99±152,41 ns
SAP [ug/ml]	290,82±77,53 ns	307,65±32,18 ns
CRP [ug/ml]	7,59± 1,82 a	13,25±2,25 b
AGP [ng/ml]	312,97±63,92 a	427,36±27,43 b
A2M [mg/ml]	4,73±0,80 ns	4,92±0,54 ns

SAP Serum Amyloid Protein/Pentraxin-2, CRP, C-Reactive Protein, AGP, Alpha-1 Acid Glycoprotein; A2M, Alpha-2 Macroglobulin in apoE/LDLR$^{-/-}$ mice fed experimental diets. Data are means ± SD. The means bearing different letters were significantly different ($p<0.05$).

Effects of LCHP diet on fatty acid composition

In our study it was observed that dietary treatments had significant effects on proportions of fatty acids in liver (Table 1B). It was observed that saturated fatty acids as well as monounsaturated fatty acids were significantly increased, whereas polyunsaturated fatty acids were significantly

decreased after LCHP feeding. Additionally, LCHP significantly increased index of hepatic SCD-1 in mice as compared to control animals.

Figure 3C
Area of lumen (%) in mice fed: Control - AIN-93G and LCHP (Low Carbohydrate High Protein) diets

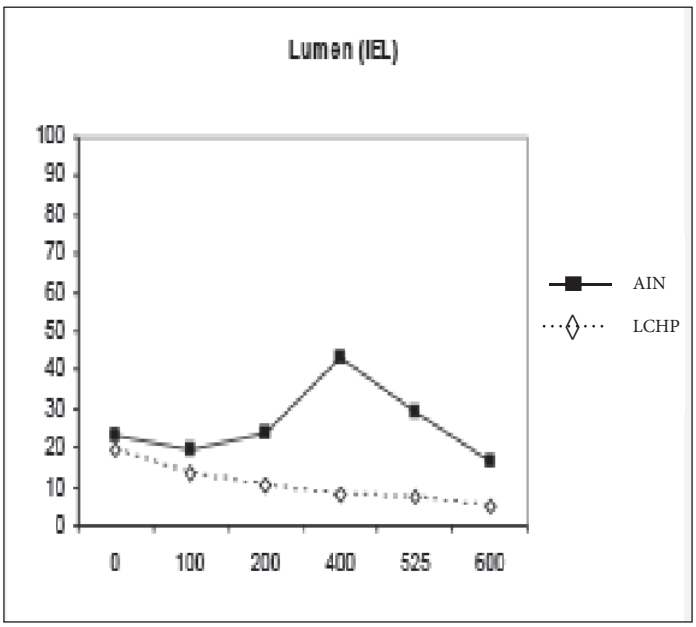


Figure 3D
Area of lipid core (%) in mice fed: Control - AIN-93G and LCHP (Low Carbohydrate High Protein) diets. The means bearing different letters were significantly different (P< 0.05)

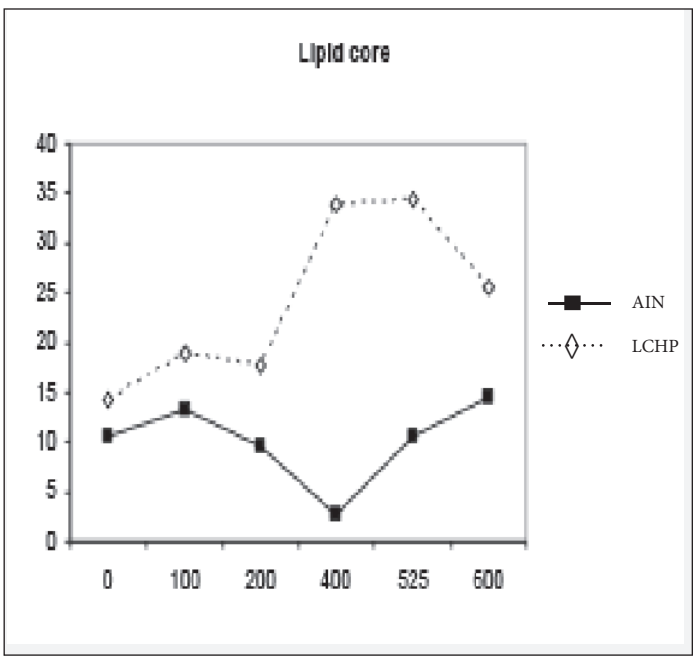


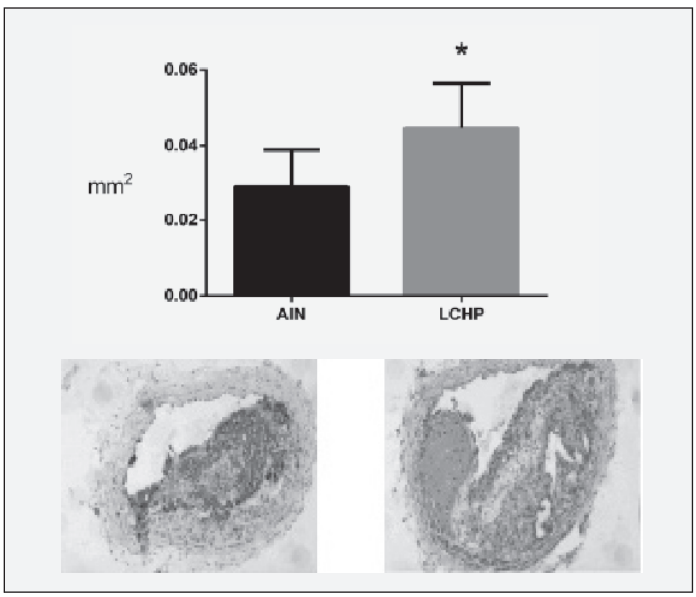
Table 3
Effects of LCHP diets on bodyweight, liver and kidney weight and their function biomarkers in plasma in mice's fed: Control - AIN-93G; LCHP (Low Carbohydrate High Protein) diets. The means bearing different letters were significantly different (p<0.05)

	AIN	LCHP
Initial body weight	22.2±1.9	22.2±0.9
Final body weight	21.2±2.4	21.9±3.1
Liver weight [g/100g b.w.]	4.7±0.5^a	5.1±0.4^b
Kidney weight [g/100g b.w.]	1.26±0.08^a	1.50±0.04^b
ALT (U/L)	83.50±64.28 ns	97.53±105.29 ns
AST (U/L)	279.32±117.39 ns	292.36±275.47 ns
LDH (U/L)	320.67±102.27 ns	257.00±159.94 ns
Urea	8.64±1.18^a	12.52±1.14^b
Creatinine clearance	87.15±53.4^a	407.75±340.5^b
Protein in urine (g/day)	1.6±0.8 ns	2.6±1.2 ns

Effects of LCHP diet on expression of ACO, FAS and SREBP-1 in the liver

Expression of genes were detected in the animal's liver (Table 1C). The expression of ACO decreased in LCHP diet. Additionally, in mice fed LCHP diet the level of FAS significantly increased. The expression of transcription factors gene like SREBP-1 (FAS) were detected. Mice fed the LCHP diet showed a slightly higher level of SREBP-1 mRNA compared to control mice.

Figure 3E
Area of macrophages (mm^2) in mice fed: Control - AIN-93G and LCHP (Low Carbohydrate High Protein) diets



Effects of LCHP diet on the plasma inflammatory markers

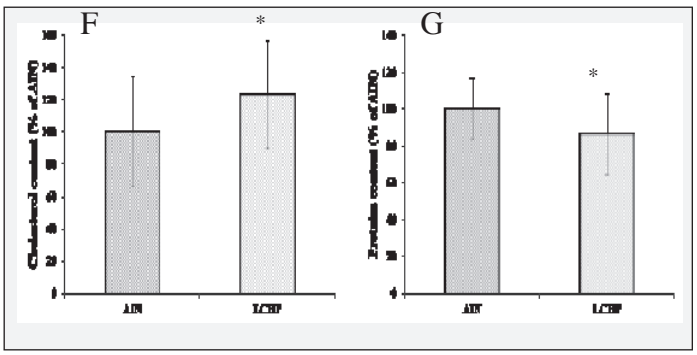
The effects of dietary treatments on the inflammatory markers are shown in Table 2. The most evident effect of dietary treatments was the significant increase in AGP and CRP level in mice fed LCHP diet as compared to controls ($P<0.05$). Additionally, A2M [mg/ml], haptoglobin [ug/ml] and SAP [ug/ml] tended to increase.

Effects of LCHP diets on bodyweight, liver and kidney weight

Initial bodyweights of mice were matched between groups of apoE/LDLR$^{-/-}$ mice prior to feeding on experimental diets. No significant differences in final body weight were observed. Liver's and kidney's weight significantly increased in LCHP fed mice (Table 3).

Figure 3F and 3G

Chemical composition changes of cholesterol (F) and proteins (G) in BCA of apoE/LDLR$^{-/-}$ mice fed Control AIN diet and LCHP diet as assessed by FT-IR spectroscopy ($p<0.01$, Mann-Whitney-Wilcoxon test)



Effects of LCHP diet on liver and kidney function

No significant differences in ALT, AST, LDH were observed between groups (Table 3). LCHP diet significantly increased creatinine clearance and urea concentration in plasma. Protein level in urine tended to increase.

Effects of LCHP diets on the mice's survival

Animals fed control diet lived significantly longer (about 12 months) than did mice fed the low carbohydrate high protein diet (Figure 4).

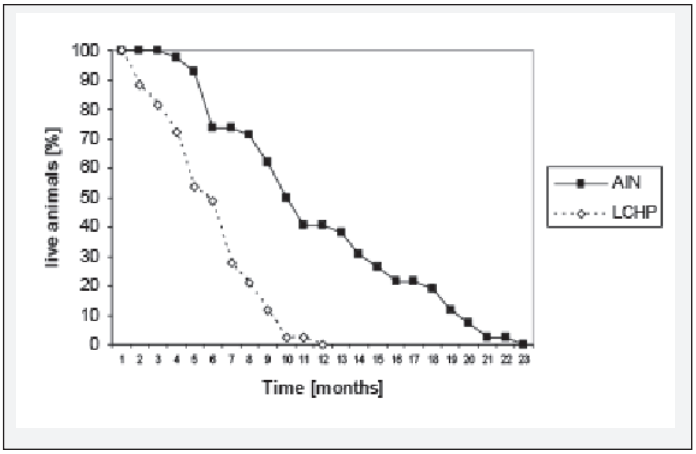
Discussion

In the present work we characterized pro-atherogenic effects of low carbohydrate high protein (LCHP) diet-induced in apoE/LDLR$^{-/-}$ mice and demonstrated that LCHP diet induced atherogenesis in apoE/LDLR$^{-/-}$ mice is linked to the profound increase in LDL and VLDL cholesterol, VLDL triglycerides, increased SCD-1 index, as well as liver SREBP-1 up-regulation, and systemic inflammation.

Our analysis of atherosclerosis was focused on atherosclerotic plaques in brachiocephalic artery (BCA) that unlike the aortic lesions more closely resemble those of human atherosclerosis (12). We found that in LCHP-fed apoE/LDL$^{-/-}$ mice atherosclerotic plaques in BCA covered larger area with more numerous macrophages, increased area of lipid core and increased number of buried fibrous caps. Our results confirms the results reported previously by Foo et al. (6) and by us (7) but extend them pointing to important role of cholesterol and triglycerides-driven mechanisms in pro-atherogenic effects of LCHP diet that favors the formation of more vulnerable plaques.

Figure 4

Survival of mice fed: Control - AIN-93G; LCHP (Low Carbohydrate High Protein) diets



The term vulnerable has been used in various reports with various definitions (13) and different markers of vulnerability at the plaque/artery level. Although there is an ongoing debate as to the occurrence of unstable plaques in hyperlipidemic mice, the phenotype of vulnerable plaque involve Morphology/Structure markers such as: plaque cap thickness, plaque lipid core size, collagen content versus lipid content and Activity/Function markers are such as: inflammatory plaque burden, intra-plaque hemorrhage, matrix-digesting enzyme activity in the cap (MMPs 2, 3, 9, etc). Additionally, number of burried fibrous cap was frequently used as a markers of plaque instability. In the present work we identified that in BCA from apoE/LDLR$^{-/-}$ mice fed LCHP diet plaque volume was increased, as well as lipid core area, cap thickness was decreased, with abundant macrophage accumulation and evidence for numerous burried caps. These results support the notion that LCHP diet not only activate atherogenesis but may also favor formation of plaques with high vulnerability index. The shorted survival of apoE/LDL$^{-/-}$ mice fed LCHP diet could be linked to thrombotic complication of atherosclerosis. On the other hand, shorten live span of LCHP fed mice may be due to general detrimental effects of LCHP e.g. on kidney function (14).

An important finding of this study was the identification

of the possible pro-atherogenic mechanisms of LCHP diet including first of all atherogenic lipid profile (increased cholesterol contents in LDL and VLDL fractions and decreased cholesterol level in HDL fraction), as well as systemic inflammation. Foo at al. (6) observed an increase in serum levels of nonesterified fatty acids (NEFAs) in mice fed LCHP diet. There is an evidence that plasma nonesterified fatty acids (NEFA) can mediate many adverse metabolic effects, most notably insulin resistance (15). However, no effect of LCHP diet on insulin level and fasting glucose were observed by Foo et al. (6). In our work (data not shown) we observed elevated fasting glucose in LCHP-fed mice but postprandial response to glucose challenge was largely preserve suggesting that glucose uptake and insulin sensitivity in skeletal muscle are preserved but rather impaired in the liver.

In the present work we demonstrated that LCHP diet had a profound effect on cholesterol distribution and lipoproteins profile. It is worth to stress that in mice fed Western diet where the amount of fat was the same as in LCHP diet, in plasma cholesterol sum was similar (2017 vs 2244mg/dL) but distribution among lipoproteins differed significantly (unpublished results). The cholesterol concentration in HDL fraction was significantly higher in mice fed WD as compared to LCHP (40,7 vs 15.2mg/dL). However cholesterol concentration in VLDL fraction was higher in LCHP diet fed apoE/LDLR$^{-/-}$ mice (1707mg/dL), than in WD fed mice (1328mg/dL). In contrast, cholesterol contents in LDL fraction was slightly higher for WD as compared with LCHP diet (648.4 vs 522.7mg/dL).

These results suggest that LCHP diet elevates concentration of pro-atherogenic lipid profile, involving mainly VLDL and possibly also chylomicrones, and these changes contribute to LCHP-induced atherogenesis.

There are a couple of possible mechanisms by which LCHP could modify lipid homeostasis. The high total fat/ saturated fat content was similar in LCHP diet and in WD and could not explain the difference in lipoprotein profile in LCHP fed apoE/LDLR$^{-/-}$ mice. On the other hand, it could well be that high protein diet containing high content of essential amino acids such as methionine, lysine, and tryptophan resulted in modified plasma and lipoprotein cholesterol (16). However, the most likely explanation is linked to the hypercholesteremic response to protein rich diet as has been previously suggested by Vahouny et al. (16). Significant increase in cholesterol absorption, a decreased conversion of cholesterol to bile acids and decreased output of fecal acidic steroids were all proposed to be involved. The hypertriglyceridemic response to casein-based diets has been suggested to be specifically due to increased hepatic production of very low density lipoproteins (17) or/and slower plasma clearance of the triglyceride-containing lipoprotein involving both intestinal and extraintestinal mechanisms (17). In the present work we demonstrated that VLDL in LCHP fed apoE/LDLR$^{-/-}$ mice was not only characterized by increased cholesterol contents but also increased triglycerides contents. Importantly, atherosclerotic plaques in BCA from LCHP fed apoE/LDLR$^{-/-}$ mice displayed higher cholesterol contents as evidenced by FTIR-based analysis, further supporting the importance of cholesterol-driven mechanisms in pro-atherogenic effects of LCHP diet.

It was shown, that LCHP can modulate lipid synthesis and accumulation by increasing SCD-1 activity. Apart from stable isotope techniques to directly determine SCD-1 desaturation activity *in vivo*, the ratios of SCD-1 product and precursor fatty acids (18:1n9/18:0 and 16:1n7/16:0) have been used as a surrogate of enzyme activity. SCD-1 catalyses the rate-limiting step in the cellular biosynthesis of monounsaturated fatty acid primarily oleate (18:1) and palmitoleate (16:1) from stearic (18:0) and palmitic (16:0) acid. These fatty acid (oleate and palmitoleate) can be incorporated into and stored as TG in the liver. Activation of SCD-1 is associated with decreased β-oxidation as well as seems to be the case in LCHP-fed animals.

The absorption of lipids in casein-fed rats would result in a more rapid influx of large chylomicrons and very low density lipoproteins into the circulation. Indeed, Vahouny et al (17) suggested, that this, if coupled with diminished plasma clearance rates of chylomicrons or their remnants, could result in a transient accumulation of triglyceride-containing lipoprotein in the circulation of casein-fed rats.

In the present work we also showed that LCHP diet sustained upregulation of genes that encode for enzymes involved in *de novo* lipogenesis, including fatty acid synthase (FAS) as well as increased sterol responsive element-binding proteins (SREBPs). These transcription factors increase the cholesterol homeostasis controlling genes, such as LDL receptor, lipoprotein lipase, and cholesterol 7α-hydroxylase (18).

In the light of the cholesterol-driven mechanisms of atherogenesis of LCHP diet in apoE/LDLR-/- mice it is not surprising that LCHP diet induced a significant increase in acute phase proteins including AGP and CRP and tended to increased A2M, haptoglobin and SAP in mice as compared to controls ($P<0.05$). Interestingly, recent studies suggested that C-reactive protein (CRP), the classical acute phase plasma protein, which is an exquisitely sensitive, nonspecific marker of inflammation binds the phosphocholine of oxidized low density lipoprotein, is not a out biomarker of inflammation but is involved in the atherogenesis as it but up-regulates the expression of adhesion molecules in endothelial cells, increases low density lipoprotein uptake into macrophages, inhibits endothelial nitric-oxide synthase expression in aortic endothelial cells and increases plasminogen activator inhibitor-1 expression and activity (19).

Finally, we showed here that LCHP diet induced a modest uremia and compensatory increase in creatinine clearance. Uremia and even mild renal dysfunction have been reported to cause a dramatic increase in plaque size and aggressive

morphology (foam cell rich soft plaques) in apoE$^{-/-}$ mice (20). High uremia, which has been associated with a prooxidative and pro-inflammatory condition, and this phenomena could also play a role in pro-atherogenic effects of LCHP diet.

In summary, here we observed that LCHP diet-induced atherogenesis in apoE/LDLR$^{-/-}$ mice is associated with cholesterol-driven mechanisms involving increased VLDL and LDL cholesterol, increased VLDL triglycerides, and systemic inflammation.

Conflict of interest: We wish to confirm that there are no known conflicts of interest associated with this publication and there has been no significant financial support for this work that could have influenced its outcome.

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