Oats *(Avena sativa)* reduce atherogenesis in LDL-receptor deficient mice.

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Supplementary material: Expanded Material and Methods, 2 tables

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ABSTRACT

Aim: The cholesterol-lowering properties of oats, largely ascribed to its contents of soluble fibers, beta-glucans, are well established, whereas effects on atherogenesis are less well elucidated. Oats also contains components with reported antioxidant and anti-inflammatory effects that may affect atherogenesis. In this work we examined effects of oat bran on plasma cholesterol, markers of inflammation, eNOS expression and development of atherosclerosis in LDL-receptor-deficient (LDLr$^{-/-}$) mice.

Methods and results: Female LDLr$^{-/-}$ mice were fed Western diet ± oat bran. Two concentrations of oat bran (40 and 27%) were compared regarding effects on plasma lipids. There was a dose-dependent reduction of plasma cholesterol by 42 and 20 % with 40 and 27 % oat bran, respectively. Both concentrations also lowered plasma triglycerides (by 45 and 33 %) and relative levels of plasma LDL+VLDL. The reduction of plasma lipids was accompanied by increased faecal excretion of cholesterol and bile acids. Oat bran (40%) efficiently reduced atherosclerotic lesion area in the descending aorta (-77%) and aortic root (-33%). Plasma levels of fibrinogen and soluble vascular cell adhesion molecule-1 (VCAM-1) were significantly lower, and immunofluorescence of aortic sections revealed a 75% lower expression of VCAM-1 in oat-fed mice. The expression of eNOS protein in the aortic wall was increased in mice fed oat bran.

Conclusions. Oat bran supplemented to a Western diet lowers plasma cholesterol, reduces levels of some inflammatory markers, increases eNOS expression and inhibits atherosclerotic lesion development in LDLr$^{-/-}$ mice. It remains to be investigated which components in oats contribute to these effects.

1. Introduction

Atherosclerosis is a chronic inflammatory process involving immunomodulatory compounds, immune cells and blood lipids[1]. Elevated plasma concentrations of total or low density lipoprotein (LDL) cholesterol are powerful risk factors for atherosclerosis, while high concentrations of high density lipoprotein (HDL) cholesterol or a low LDL / HDL ratio are considered to protect against vascular disease. Atherosclerosis is expected to be associated with increased levels of inflammatory markers, present in the lesions as well as in blood plasma[2]. Moreover, endothelial dysfunction, with reduced NO production, has emerged as an important underlying factor in the pathogenesis of vascular disease. Nutritional factors are connected with the development of atherosclerosis, and efforts to reduce the rapidly spreading adverse effects associated with Western eating habits are highly needed[3].

During the last decades the role of dietary fibers in the control of lipid and lipoprotein metabolism has attracted much attention. Oats and soluble fibers from oats, beta-glucans, have been shown to reduce plasma cholesterol levels in man[4-6]. In vitro experiments point in the direction that also other components in oats, such as vitamin E (α-tocopherol), phenolic acids, flavonoids and sterols may have antiatherosclerotic properties by exerting anti-oxidative and anti-inflammatory effects, and by retaining endothelial function[7-9]. This suggests that increased consumption of oat-based food products may be beneficial in reducing the incidence and severity of atherosclerosis in the general population. More knowledge is however needed, including evaluation of which properties of oats are crucial for reducing atherosclerosis, as this would allow the development of optimal oat-based food components. Animal studies will be helpful for this purpose, and despite extensive studies of the lipid-lowering properties of oat products[4-6] there are few investigations directly addressing atherosclerosis development. The effects of several fiber products, including oat bran, on lipid infiltration in aortas of Syrian hamsters fed a cholesterol-enriched diet have been studied in

1 relation to effects on plasma lipids[10,11], but inflammatory markers or endothelial functions

2 were not investigated. Mice are advantageous in comparison with other animal experimental

3 models, in view of the large number of genetic variants and extensive information on

4 atherogenic mechanisms available in this species. We have shown that oat bran reduces total

5 and LDL/HDL ratio in C57BL/6 mice[12]. For the study of atherosclerosis, mouse models

6 with propensity for hyperlipidemia offer the possibility to study lesion development over

7 limited time periods.

8   The most common lipoprotein pattern in humans with coronary artery disease consists of

9 elevated plasma levels of LDL with or without increases in VLDL, and decreased

10 HDL/(LDL+VLDL) ratio[1]. Such a lipoprotein pattern is seen in LDL-receptor knock-out

11 (LDLr$^{-/-}$) mice, which develop atherosclerotic lesions when fed a high-fat ("Western") diet

12 [13]. The present study aimed to investigate the effects of oats on atherosclerotic lesion

13 formation, markers of inflammation, endothelial function and plasma lipids in LDLr$^{-/-}$ mice.

14 Oat bran was used here to establish effects of unprocessed oats. In addition to the mechanistic

15 information obtained, the results will be useful as a reference for further studies involving

16 isolated oat components.

17

2. Materials and methods

2.1. Mice

20   Female LDL-receptor-deficient homozygous (LDLr$^{-/-}$) mice were purchased from Charles

21 River Laboratories (Sulzfeld, Germany). All experiments were approved by the Malmö/Lund

22 regional ethical committee for laboratory animals (M86-05) and followed national guidelines

23 for the care and use of animals. At 8-10 weeks of age, the mice were randomly divided into

24 two groups of 20 animals. One group was fed Western diet supplemented with 40% oat bran

1   and the other group control Western diet for 16 weeks. In a separate, 4-week, follow-up

2   experiment we evaluated the effects on plasma cholesterol of a lower concentration of 27%

3   oat bran, and the expression of eNOS in the aortic arch.

4   *2.2. Diets*

5   A Western diet (Research Diets Inc., NJ, USA) was supplemented with 40 % or 27% oat

6   bran (wt/wt) at the expense of casein, corn starch, sucrose and butter (please see online Tables

7   S1 and S2). Control and oat bran diets were formulated to keep fiber contents, energy and

8   macronutrients equal, with 16% protein, 42% carbohydrates, 42% fat (energy percent) and

9   0.05% cholesterol in both control and oat bran diets. In the control diets oat fibers were

10 replaced by microcrystalline cellulose, see[12] for details. Feed consumption was determined

11 per cage over 1-week periods and expressed as gram consumed per mouse per day.

12 *2.3. Tissue sampling and analyses*

13 Blood samples drawn from vena saphena at baseline and at weeks 1, 2, 4, 8, 12 and 16

14 were collected after 4 h fasting. At week 16 all the faeces excreted during 24 h were collected

15 from each cage (10 mice/cage). At the end of the study mice were anaesthetized and perfusion

16 fixed. The aorta was mounted *en face* and stained with Oil Red O. Lesion areas were

17 quantitated using Image ProPlus 4.5 Software (MediaCybernetics, Inc. MD, USA). Cryo

18 sections of the aortic root were used for immunofluorescence detection of VCAM-1 and for

19 lesion analysis with Oil Red O and hematoxylin staining, respectively. The caecum with its

20 contents was removed, emptied and weighed. For analysis of eNOS by Western blot, mice

21 were sacrificed by cervical dislocation and the aorta dissected free and frozen at -80°C.

22 *2.4. Plasma analyses*

23 Total plasma cholesterol and triglyceride levels were determined enzymatically. Plasma

24 lipoproteins were separated by electrophoresis in 0.8% agarose gels in barbital buffer as

5

1 described previously [12]. The relative lipid distribution between HDL and VLDL+LDL was

2 calculated. This method does not reveal LDL cholesterol directly, but rather shows the

3 distribution of total lipids (cholesterol, triglycerides and phospholipids) among the different

4 lipoproteins. Commercially available murine ELISA kits were used for measuring plasma

5 levels of inflammatory markers serum amyloid A (SAA), fibrinogen, interleukin-6 (IL-6) and

6 soluble VCAM-1 (sVCAM-1) in plasma.

7 *2.5. Immunofluorescence*

8 Cryo sections of the aortic root were incubated with primary rat-anti-mouse VCAM-1

9 antibodies (Chemicon) and then with secondary Cy5 donkey-anti-rat antibodies (Santa Cruz).

10 Nuclei were counter-stained with SYTOX® Green (Molecular Probes, (Invitrogen) USA). A

11 Zeiss LSM 5 Pascal laser scanning confocal microscope was used to quantitate the VCAM-1

12 stained area relative to the total vessel wall area.

13 *2.6. Western blot*

14 Proteins were separated with SDS-PAGE (10.5-14% gels) and transferred to a nitrocellulose

15 membrane. The membranes were incubated with an antibody against eNOS (BD Transduction

16 Lab.), detected with chemiluminescence (West Femto, Nordic Biolab) and analyzed with

17 Quantity One software (BioRad). Intensities of eNOS-bands were normalized to

18 corresponding parts of the gel stained with Comassie Blue following transfer as loading

19 control.

20 *2.7. Statistical analysis*

21 Data are expressed as mean ± SEM if not otherwise stated. Differences between oat bran

22 and control were compared by Student's t-test for unpaired data, while paired t-tests were

23 used for within-group changes from baseline. In lesion analysis of flat preparations data

1   median values were analyzed with Mann-Whitney U-test. Values of P<0.05 were considered

2   statistically significant.

3   An extended section 2 is presented as Supplementary data.

4

5   **3. Results**

6   *3.1. Body weight and feed intake*

7   There was no difference in feed intake between oat bran groups and control groups, and all

8   groups of mice gained body weight throughout the study, with a somewhat greater increase in

9   the oat bran than in the control group (Table 1). Greater weight gain in oat-fed animals has

10   earlier been observed in rats[14].

11   *3.2. Oat bran reduces plasma lipids in LDLr$^{-/-}$ mice.*

12   Western diet induced a prominent increase in plasma cholesterol. Cholesterol levels of

13   mice fed 40% oat bran were approximately 40 % lower than those of controls throughout the

14   study (Fig. 1A). Oat bran (27%) led to a reduction of plasma cholesterol by 20% (Fig. 1B).

15   Plasma triglycerides were more than doubled when mice were fed the Western diet, but were

16   reduced by 45% and 33% by 40 and 27% oat bran, respectively (Fig. 1C).

17   Although the Western diet greatly increased total cholesterol, there were no dramatic

18   changes in lipoprotein profiles in the LDLr$^{-/-}$ mice. In control groups the relative LDL+VLDL

19   lipid levels were higher at the end of the studies (16 and 4 weeks) compared to baseline,

20   whereas there were no statistically significant differences from baseline in the oat bran

21   groups. At the end of the studies the relative amounts of LDL+VLDL lipids were significantly

22   lower in the oat-fed mice (both with 40% and 27% oat bran) than in controls (Fig. 1D).

23

1 *3.3. Cholesterol and bile acid excretion and caecum contents*

2   There was an almost fourfold increase in faecal cholesterol excretion (Fig. 1D) and a 40%

3 increase in faecal bile acid excretion (Fig. 1E) in mice fed oat bran (40%), although the total

4 amount of faeces excreted did not differ: 2.47 and 2.51 g/mouse/24h in control and oat-fed

5 mice, respectively. The mice fed 40% oat bran had almost twice as much caecum contents as

6 the control mice and also had higher caecum wall wet weights. The lower concentration of oat

7 bran (27%) did however not cause any statistically significant changes in either caecum

8 content or caecum wall weight (Table 1).

9 *3.4. Effects of oat bran on inflammation markers*

10   The acute-phase proteins fibrinogen and serum amyloid A (SAA) as well as IL-6 and

11 sVCAM-1 were analyzed in plasma samples after 16 weeks on experimental diet. Fibrinogen

12 was significantly lower in mice fed oat bran (40%) than in control mice (P<0.001; Fig. 2A),

13 whereas SAA tended to be lower but no significant difference was found (P=0.09; Fig. 2B).

14 Oat bran (40%) did not alter plasma concentrations of IL-6 (Fig. 1C). The sVCAM-1

15 concentration was reduced by 10% and 7% in mice fed 40 and 27% oat bran, respectively

16 (P<0.05; Fig. 2D and E). Confocal immunofluorescence analysis of cryosections of the aortic

17 root revealed that oat bran (40%) significantly reduced the expression of the adhesion

18 molecule VCAM-1 in the vessel wall (Fig. 3A and B).

19 *3.5. Effect of 27% oat bran on eNOS expression*

20   The eNOS expression in the aorta was determined after 4 weeks on an experimental diet

21 containing 27% oat bran or control fiber. Western blot analysis of the aortic arch revealed a

22 greater eNOS expression in mice fed oat bran (Fig. 3C).

23

24

1 *3.6. Oat bran reduces atherosclerotic lesion development.*

2   The effect of oats on atherosclerosis was evaluated in *en face* preparations of the

3   descending aorta, and in cryosections of the aortic root, stained by Oil Red O (Fig. 4A andB).

4   Oat bran (40%) diet significantly reduced lipid infiltration in the descending aorta, with

5   median value for lesion area of 0.19% of total aortic area vs. 0.82% in control mice (Fig. 4A).

6   The control and oat-fed groups each contained three outliers with elevated lesion areas,

7   whereof one in the oat group had an excessively large lesion area. These outliers did not differ

8   from the rest of the mice in any other investigated respect and were all included in the results

9   and statistical analysis. The total lesion area in the aortic root was also significantly smaller in

10 mice fed oat bran (40%) (Fig. 4B).

11

4. Discussion

13   Effects of two doses of oat bran were evaluated in this work. We started with a high (40%)

14 dose to ascertain an effect on plasma lipids and on atherosclerosis to establish the model. A

15 lower dose (27%) was then used in a shorter follow-up study with plasma lipids as main end-

16 point. Results suggest that oat bran causes a dose-dependent reduction of plasma cholesterol

17 in LDLr$^{-/-}$ mice. Oat bran also reduces atherosclerotic plaque development and some

18 inflammatory markers, and furthermore increases bile acid excretion and the expression of

19 eNOS in the aortic wall of LDLr$^{-/-}$ mice. The study extends our recently reported observations

20 on plasma lipids in C57BL/6 mice[12] by directly demonstrating antiatherogenic effects of

21 oats in a hyperlipidemic mouse model.

22   A smaller atherosclerotic lesion area after 16 weeks of Western diet was found in the oat-

23 fed mice relative to the control group. Reduced levels of plasma cholesterol in the oat-fed

24 mice are probably the most important reason for the reduced atherosclerotic lesion area.

1 However, oat bran contains components that may exert antioxidative and anti-inflammatory

2 effects, such as vitamin E (α-tocopherol), phenolic acids, flavonoids and sterols[7]. The

3 summed effect of these compounds could possibly counteract atherosclerotic lesion

4 development in addition to the effect of lowered plasma lipids.

5   Atherosclerosis is a disorder driven by inflammation, and the molecular mediators of

6 inflammation also serve as inflammatory markers to predict future cardiovascular risk[2].

7 Examples of such markers include the plasma acute-phase proteins C-reactive protein (CRP),

8 SAA and fibrinogen, as well as cytokines such as IL-6, IL-1 and TNFα[15]. In LDLr^{-/-} mice

9 fed high-fat diet the prevalence of atherosclerotic lesions correlates with increased plasma

10 concentrations of SAA[16], and elevated levels of the adhesion molecules VCAM-1 and

11 ICAM-1 on endothelial and vascular smooth muscle cells have been found in

12 atherosclerosis[17]. Dietary factors can influence systemic inflammation. For example,

13 human consumption of whole grains has been shown to correlate with reduced CRP levels as

14 well as with reduced progression of coronary atherosclerosis[18,19], and reduction of

15 adhesion molecule expression by flaxseed dietary fibers was associated with reduced

16 atherosclerosis in LDLr^{-/-} mice on a high-fat diet[17]. Regarding oats, very little *in vivo*

17 evidence on anti-inflammatory effects exists. In a recent human study, consumption of oat

18 beta-glucans was shown to effectively reduce LDL cholesterol but no influence on

19 inflammatory markers could be demonstrated[20,21].

20   In the present *in vivo* study, plasma markers of inflammation (sVCAM-1 and fibrinogen),

21 as well as the expression of VCAM-1 in lesion areas, were reduced in the oat-fed mice. There

22 was also a tendency to reduced levels of plasma SAA. We did not see an effect on plasma

23 levels of IL-6, but cannot exclude that different results would have been obtained at other

24 time points. Possibly, plasma levels of cytokines and other inflammatory markers are more

25 affected postprandially than in a fasted state as studied here. Avenanthramides, oat-specific

1   phenolic antioxidants, have been shown to reduce levels of VCAM-1, ICAM-1 and IL-6 in

2   endothelial cells *in vitro*[8], and other antioxidants (vitamin E, vitamin C and beta-carotene)

3   have been shown to reduce levels of sVCAM-1 in wild-type and ApoE*3 Leiden mice[22].

4   Therefore avenanthramides, E-vitamins and other phenolic compounds present in oat bran,

5   although in low concentrations, may have contributed to an anti-inflammatory effect. We can

6   however not rule out that the reduced levels of blood lipids in the oat-fed mice was an

7   important factor for the reduced inflammation observed, since hyperlipidemia *per se* elicits

8   pro-inflammatory responses[23].

9   Generation of nitric oxide (NO) by endothelial nitric oxide synthase (eNOS) is known to

10   protect against vascular disease, and eNOS/ApoE double knockout mice on a high-fat diet

11   show accelerated atherosclerosis[24]. *In vitro* studies have shown that polyphenols from oats

12   can increase the expression of eNOS in endothelial cells[9]. In the present *in vivo* study, we

13   found increased eNOS protein levels in the aortic arch of oat-fed mice compared with

14   controls. This suggests that oats are indeed able to improve endothelial function. Previous

15   studies in LDLr$^{-/-}$ mice have indicated that reduction of inflammation by antioxidants can

16   limit atherosclerosis and increase NO production at constant plasma lipid levels[25]. Further

17   studies are needed to address this issue for oats, with its inherent effects on plasma lipids.

18   Possible approaches would involve either modification of diets to produce similar lipoprotein

19   levels in the absence and presence of oats, or extraction of constituents with presumed anti-

20   inflammatory properties from the oat preparation.

21   One of the postulated mechanisms for the cholesterol-lowering effect of oats is that the

22   beta-glucans either bind bile acids or create a viscous layer at the absorption surface in the

23   small intestine and thereby decrease intestinal uptake of dietary cholesterol and reabsorption

24   of bile acids[26,27]. Accordingly, we observed increases in faecal excretion of both

25   cholesterol and bile acids after oat consumption, as has been found previously in C57BL/6

1   mice[12], hamsters[11] and humans[28,29]. The increase in bowel contents seen after oat

2   feeding has been attributed to greater water binding and is thought to contribute to the altered

3   viscosity of the intestinal contents[26]. Findings in the LDLr$^{-/-}$ mice are thus consistent with

4   mechanisms thought to account for the effects of oats in the intestinal lumen, although their

5   lack of LDL-receptors reduces cholesterol clearance from plasma.

6   The LDLr$^{-/-}$ mice responded to Western diet with a prominent increase of plasma

7   triglycerides, which was significantly reduced by oat bran. Increased serum triglyceride levels

8   are related to atherosclerosis independently of serum levels of HDL and LDL and the

9   triglyceride-rich remnant lipoproteins produced by hydrolysis of VLDL and chylomicrons are

10   highly atherogenic[30]. Correct function of the LDL-receptor is essential for the clearance of

11   lipoprotein remnants although other types of lipoprotein receptors, such as the LDLr related

12   protein (LRP), have been suggested to participate[31]. Therefore, effects of intestinal

13   triglyceride uptake on plasma levels are expected to be amplified in the LDLr$^{-/-}$ mouse.

14   Accordingly, the present results suggest that intestinal triglyceride uptake is reduced by the

15   oat bran diet.

16   The results of this study in LDLr$^{-/-}$ mice show that oat bran reduces plasma levels of total

17   cholesterol, triglycerides, LDL+VLDL lipoproteins, and some important inflammation

18   markers. It also reduces adhesion molecule expression and increases eNOS expression in the

19   aortic wall. Most importantly, oat bran not only reduces plasma cholesterol, but also inhibits

20   atherogenesis. A limitation with the present study is that the use of whole oat bran does not

21   allow us to see if the reduced atherogenesis and inflammation represent direct effects of

22   components present in oats or if they result entirely from the lowered plasma cholesterol *per*

23   *se.* This has to be addressed in future work, where the mouse model will be an important tool.

24   Development of new oat-based products and evaluation of their effects on atherogenesis is

1 expected to lead to improved public health by providing new approaches to health-beneficial

2 dietary habits.

3

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Figure legends

Fig. 1. Effect of Western diet ± oat bran on plasma lipids and faecal cholesterol and bile acids. Total cholesterol vs. time after intake of 40 % (**A**) or 27 % (**B**) oat bran. The triglycerides (**C**) and relative amounts of LDL+VLDL (**D**) at baseline (striped bars) and at 16 weeks with 40 % oat bran, or at 4 weeks with 27 % oat bran (n=19-20). The faecal cholesterol excretion (**E**, n=6), and the faecal total bile acid excretion (**F**, n=6) in mice fed 40 % oat bran. *P<0.05, **P<0.01, ***P<0.001 vs. control, and [#]P<0.05, [##]P<0.01, [###]P<0.001 vs. baseline.

Fig. 2. Effect of oat bran on plasma inflammatory markers in LDLr^{-/-} mice. (**A**)-(**C**) Fibrinogen, SAA and IL-6 after intake of 40 % oat bran for 16 weeks. (**D**) Soluble VCAM-1 after intake of 40 or 27 % oat bran for 16 and 4 weeks, respectively (n=19-20). ***P<0.001, and *P<0.05 vs. control, ns: not significant.

Fig. 3. Effect of oat bran on VCAM-1 and eNOS expression in the aortic root. (**A**) Representative images of VCAM-1 expression (red) and SYTOX[®] Green nuclear staining in cross sections of aortic root. Scale bar: 200 μm. (**B**) Summary of VCAM-1 expression in cross sections of aortic root (n=10). (**C**) Western blot analysis of eNOS expression. Upper panel: eNOS and lower panel: corresponding part of gel stained with Comassie Blue following transfer (loading control). (**D**) Summary of Western blot data (n=16-18) ***P<0.001, **P<0.01, *P<0.05 vs. control.

Fig. 4. Oat bran (40 %) reduces atherosclerotic lesion development. (**A**) Oil Red O stained *en face* preparations of aorta and quantitative data (n=19-20, lines show median values). Images shown are composites, see Supplementary data for details. (**B**) Aortic root cross sections stained with Oil Red O and quantitation of lesion size (n=7-9). *P<0.05, **P<0.01 vs. control

TABLE

Table 1. Initial body weight, body-weight gain, feed intake, caecal content and caecum wall weight in LDLr$^{-/-}$ mice fed control or 40 % oat bran Western diet after 16 weeks, and control or 27 % oat bran Western diet after 4 weeks.

	Control 16 weeks			Oat bran 40 % 16 weesk				Control 4 weeks			Oat bran 27 % 4 weeks			
	Mean	SEM	(n)	Mean	SEM	(n)		Mean	SEM	(n)	Mean	SEM	(n)	
Initial body weight (g)	18.5	0.25	(19)	19.0	0.23	(20)	ns	20.1	0.44	(20)	20.3	0.48	(20)	ns
Body-weight gain (g)	6.6	0.46	(19)	8.4	0.56	(20)	P<0.05	1.5	0.21	(20)	2.7	0.36	(20)	P<0.01
Feed intake (g/mouse/ day)	2.2	0.03	(24)	2.2	0.04	(24)	ns	2.4	0.06	(7)	2.3	0.07	(8)	ns
Caecal content (g)	0.12	0.01	(17)	0.21	0.02	(17)	P<0.001	0.15	0.01	(10)	0.17	0.01	(10)	ns
Caecum wall weight (mg)	53.9	2.8	(19)	69.2	3.1	(20)	P<0.001	19.6	2.4	(10)	24.0	2.5	(10)	ns

ns: not significant
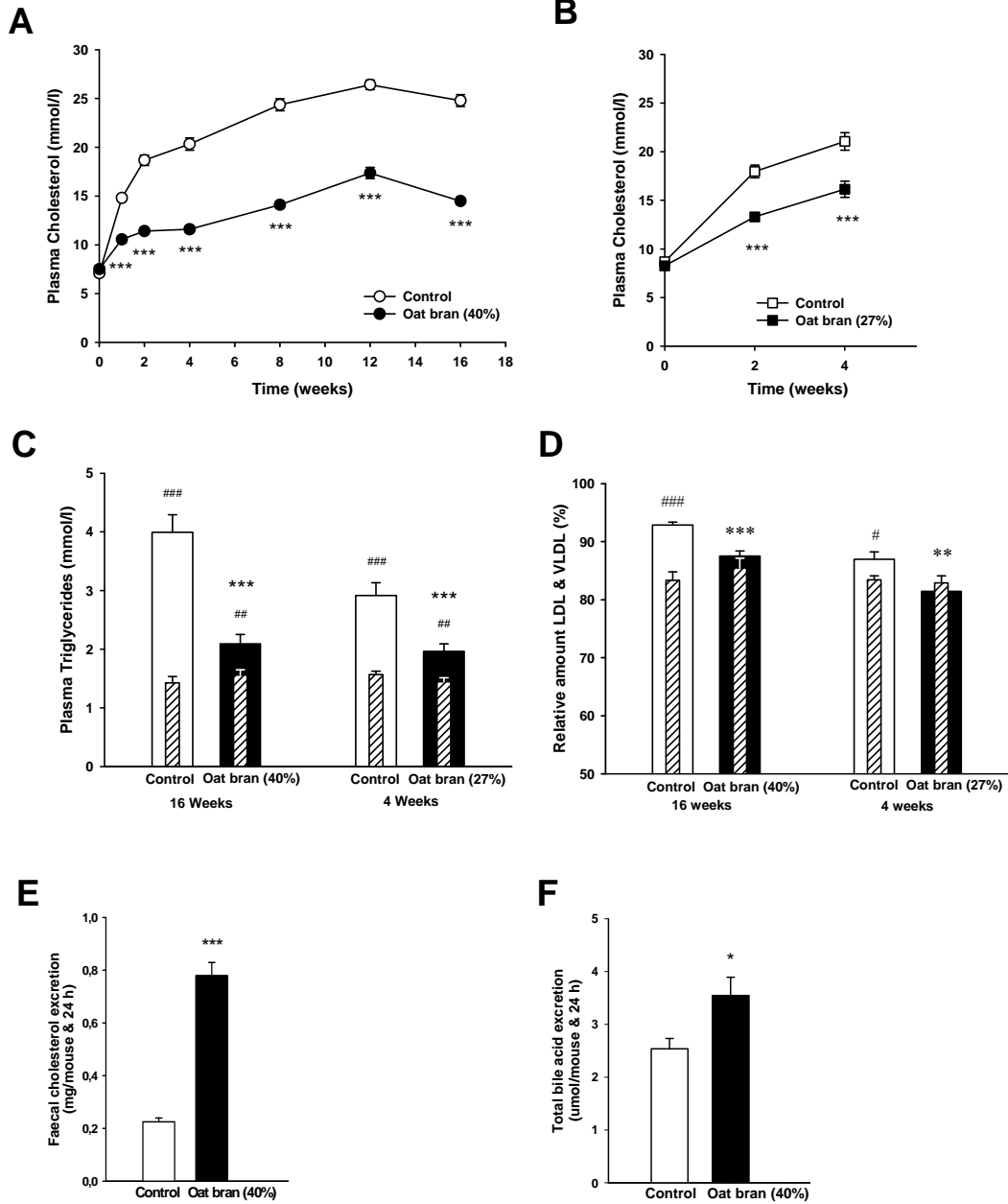
Figure 1.
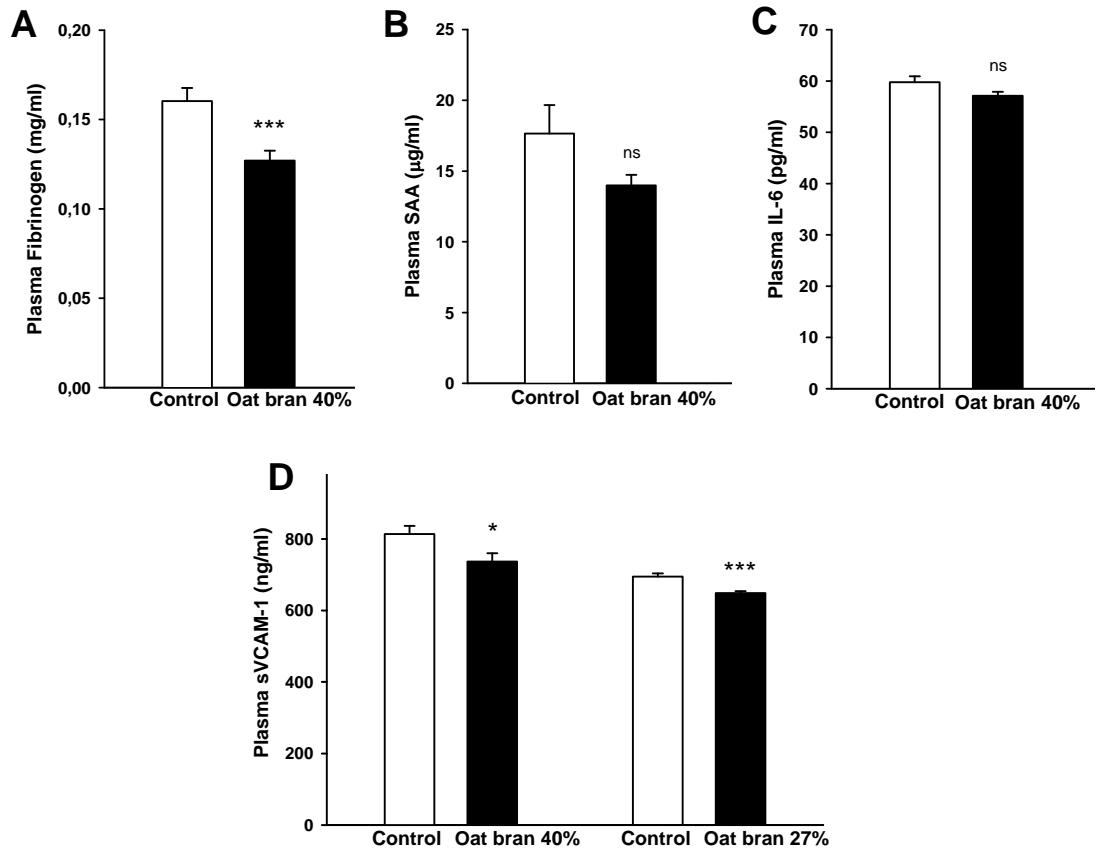
Figure 2

A
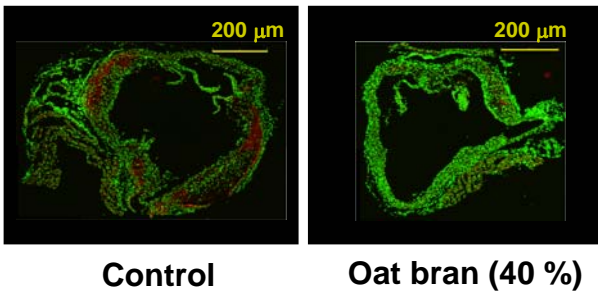
Control   Oat bran (40 %)

B

C

D

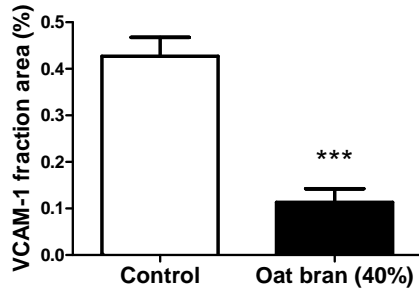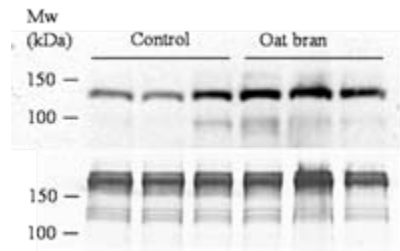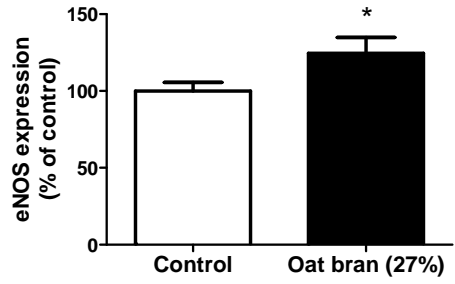**Figure 3.**

Figure 4.

Oats *(Avena sativa)* reduce atherogenesis in LDL-receptor deficient mice.

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Supplementary material

Materials and Methods

Mice

Female LDL-receptor deficient homozygous (LDLr$^{-/-}$) mice were purchased from Charles River laboratories (Sulzfeld, Germany). All experiments were approved by the Malmö/Lund regional ethical committee for laboratory animals (M86-05) and followed national guidelines for the care and use of animals. During an adaptation period of 2 weeks all mice were fed standard chow (R34, Lactamin, Sweden). At 8-10 weeks of age, the mice were randomly divided into two experimental groups, each containing 20 animals housed in cages of 10 mice. Blood samples were taken to establish baseline plasma parameter values. One group was fed Western diet supplemented with oat bran (40 %) and the other group control Western diet. The mice had free access to food and water throughout the experiment. They tolerated the studies well, but one mouse in the control group had to be sacrificed at week 12 and excluded from the study because of wounds on the hind leg. In an additional experiment we evaluated the effects on plasma cholesterol of a lower concentration of oat bran (27 %). Two groups of 20 LDLr$^{-/-}$ mice each were fed Western diets for 4 weeks, with addition of 27 % oat bran in the test diet. The aortas of these mice were used to determine the expression of eNOS in the aortic arch.

Diets

A Western diet was supplemented with 40 % or 27 % oat bran (wt/wt) at the expense of casein, corn starch, sucrose and butter (Table S1 and S2). In the control diets oat fibers were replaced by microcrystalline cellulose (Avicel® PH 101, FMC Biopolymer). Control and oat bran diets were formulated to keep fiber contents, energy and macronutrients equal, with 16 % protein, 42 % carbohydrates, 42 % fat (energy percent) and 0.05 % cholesterol in both

control and oat bran diets. Diet premixes were purchased from Research Diets Inc. (New Brunswick, NJ, USA), and melted anhydrous butter, maltodextrin, cellulose and oat bran were added in our laboratory by thorough mixing.

The oat bran used (*Avena sativa* cv. Sang, batch 1008596, Lantmännen AB, Järna, Sweden, pre-milled to a particle size less than 0.8 mm) had a total fiber content of 16 % (analyzed by Eurofins Food, Lidköping, Sweden), whereof 7.2 % was beta-glucans, determined enzymatically (Megazyme International, Wicklow, Ireland). The diet with 40 % oat bran thus contains approximately 3.0 % beta-glucans and has a total fiber content of 6.5 %, whereas the diet with 27 % oat bran contains 2 % beta-glucans and 4.4 % total fiber. In the control diets oat fibers were replaced by 6.5 % and 4.4 % microcrystalline cellulose respectively. Feed consumption was determined per cage over 1-week periods and expressed as g consumed per mouse per day.

Tissue sampling

Blood samples were drawn from vena saphena at baseline and at weeks 1, 2, 4, 8, 12 and 16. The samples were collected in EDTA-coated microvette tubes after 4 h fasting. Plasma was prepared by spinning whole blood at 5000 x g for 10 minutes at 4°C and stored at -80°C until assayed. Plasma aliquots for lipoprotein analysis were supplemented with sucrose to a final concentration of 10 % to protect the structure of the lipoproteins before freezing[1].

At week 16 all the faeces excreted during 24 h were collected from each cage (10 mice/cage). The faeces were lyophilized and weighed and then three parallel samples from each cage were analyzed for cholesterol content or total bile acids. For cholesterol measurements lipids were extracted from 0.1 g of minced faeces with 5 ml hexane:isopropanol (3:2 + 0.005% 2,6-Di-Tert-Butyl-4-Metylphenol), dried under nitrogen and reconstituted in isopropanol with 1 % Triton X-100. This lipid extract was then analyzed with the same cholesterol reagents as the plasma samples. For bile acid measurements 0.25 g faeces were minced and extracted in 5 ml of 75 % ethanol at 50 °C for 2 h (as described in Yu et al.[2] After centrifugation the extract was diluted 1:10 in water before enzymatic determination in a 96-well format using the Diazyme Colorimetric Total Bile Acids Assay Kit according to the manufacturer´s directions.

At the end of the study with 40% oat bran mice were anasthesized with rompun/ketalar and perfusion fixed with 15 ml Histochoice tissue fixative (Amresco, Ohio, USA), injected into

the left ventricle. The aorta was dissected free from connective and adipose tissue and cut open longitudinally from the iliac arteries to 1 mm below the left subclavian artery. It was then mounted *en face* on an ovalbumin-coated glass slide with the luminal side up[3]. After drying, the slide with the aorta was placed in Histochoice until staining. The aortic arch attached to the heart was placed and stored in Histochoice until embedded in OCT (Sakura Finetek, USA) and cryo-sectioned. The hearts were cut in 10 μm sections using a cryomicrotome at -22 °C. Starting when all 3 aortic valve cusps were clearly visible in one section, five sections of the aortic root at 100 μm intervals were collected from each mouse and the avarage of the lesion area was counted as n=1 in the statistical analysis. Cryo section collected like this were used for both immunfluorescence measurement of VCAM-1 and lesion analysis with Oil Red O and hematoxylin staining. The caecum with its contents was removed, emptied and weighed.

In the study with 27 % oat bran the mice were sacrificed by cervical dislocation, the aorta was removed, dissected free from connective tissue and kept frozen at -80°C before Western blot analysis.

Plasma lipids

The total plasma cholesterol and triglyceride levels were determined by enzymatic analysis with Infinity cholesterol/triglycerides Liquid Stable reagent (Thermo Trace, Noble Park, Australia). Plasma lipoproteins were separated by electrophoresis in 0.8 % agarose gels in barbital buffer[4] using a Sebia Hydragel 7 Lipoprotein(E), K20 chamber (Sebia, France). Apolipoprotein B-containing lipoproteins (LDL and very low density lipoprotein, VLDL) are separated from HDL by this method due to their individual charges. After Sudan black staining of the gels and densitometric scanning (BioRad GS 800 Calibrated Densitometer and Quantity One quantitation software) the relative amounts of HDL and VLDL+LDL were calculated from the intensity of the bands. Values of VLDL and LDL were summed since the bands are not always clearly distinguishable. This method yields relative amounts of lipoproteins in each sample.

Plasma inflammatory markers

Commercially available murine ELISA kits were used for measuring plasma levels of inflammatory markers SAA (Tridelta Development Ltd, Ireland), fibrinogen (Immunology Consultants Laboratory, Inc. Newberg, USA) IL-6 and soluble VCAM-1 (sVCAM-1; R&D Systems, Inc. Minneapolis, USA) in plasma collected after 16 weeks experimental food administration.

Oil red O staining

The aortic *en face* preparations were stained with Oil Red O as described by Brånén et al[3] and covered with a coverslip. Fatty lesions were recognizable as dark, burgundy red objects, whereas adventitial fat stained much lighter red. Images of the *en face* preparations were captured at 20x magnification (7-8 images per aorta) and analyzed with Image ProPlus 4.5 Software (MediaCybernetics, Inc. Bethesda, MD, USA). The lesion size was expressed as lesion area in percent of total aortic surface area. The outline of the aortic surface and lesions were defined manually by a blinded observer. Pictures of aorta shown in Figure 3A are composites of 7-8 aligned, non-overlapping images.

Cryo sections of the aortic root from 10 randomly selected animals from each group were stained with Oil red O and counterstained with haematoxyline. The lesion size was quantified by computer-assisted morphometric analysis and expressed as mean lesion area per section.

Analysis of VCAM-1 in aortic sections

Cryo sections of the aortic root were incubated with primary rat-anti-mouse VCAM-1 antibodies (Chemicon) 1:200 in BSA and then with secondary Cy5 donkey-anti-rat antibodies (Santa Cruz) 1:500 in BSA. Counter staining for nuclei was done with SYTOX® Green (Molecular Probes, (Invitrogen) USA) 1:3000 in PBS. A Zeiss LSM 5 Pascal laser scanning confocal microscope was used to scan 1024x1024 px 8-bit image. Detector settings were fine calibrated for each glass slide and then kept constant per slide. All images were taken using a Plan-Neofluar 10x/0.3 lens using appropriate filter sets for Cy5 and SYTOX® Green emissions.

Image analysis was done using a custom-written program implemented in MATLAB^{TM} 2007b to quantitate the pixel area of 670 nm-emitting (VCAM-1 stained) fluorescence relative

to the total vessel wall area, which was obtained by supervised thresholding. Images were run through a 3x3px, σ = 5 Gaussian filter to reduce effects of random noise. The weak autofluorescence signal detected in the 545-650 band was used to segment out tissue in the images by supervised thresholding and connected component analysis. Next, supervised thresholding was applied to generate a VCAM-1 only logic image. This image was subsequently multiplied by the tissue-segmented image. These final resultant images were used to obtain percentages of VCAM-1 signal found in the vessel wall.

Western Blot eNOS

In the additional experiment with LDLr$^{-/-}$ mice fed Western diet ± 27 % oat bran for four weeks the aortic arch were dissected free from adventitial fat and homogenized in SDS sample buffer (62.5 mM Tris-HCl, 2% SDS, 10% glycerol, 5% mercaptoethanol, 0,001% bromphenolblue and protease inhibitor coctail (Sigma). The homogenates were boiled, centrifuged and protein concentrations were determined with EZQ protein determination (Molecular Probes). Proteins were separated with SDS-PAGE (10.5-14 % gels) on a BioRad Criterion system loaded with 25 µg protein in each lane and transferred to a nitrocellulose membrane. The membranes were incubated with an antibody against eNOS (BD Transduction Lab.), detected with chemiluminiscence (West Femto, Nordic Biolab) and analysed with Quantity One software (Biorad).

Statistical Analysis

Data are expressed as mean ± SEM if not otherwise stated. Differences between oat bran and control were compared by one-tailed Student's t-test for unpaired data, while paired t-tests were used for within-group changes from baseline. In lesion analysis of flat preparations data median values were analyzed with Mann-Whitney U-test. Values of $P<0.05$ were considered statistically significant.

References

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Table S1. Formulation of the Western diets and macronutrient contents

Ingredient	Control	Oat bran (40%)
	g/kg diet	g/kg diet
Casein, 80 Mesh[*]	200	119
DL-Methionine[†]	3	5
Corn Starch	265	53
Maltodextrin	100	100
Sucrose	100	91
Cellulose	65	0
Butter, Anhydrous[‡]	200	165
Corn Oil	10	10
Mineral Mix S10026	10	10
DiCalcium Phosphate	13	13
Calcium Carbonate	5.5	5.5
Potassium Citrate, 1 H2O	16.5	16.5
Vitamin Mix V10001	10	10
Choline Bitartrate	2	2
Cholesterol[‡]	0	0.08
Oat Bran[§]	0	400
Total	1000	1000
Macronutrient contents[‖]	% energy	% energy
Protein	16	16
Carbohydrate	42	42
Fat	42	42

[*]Casein is 88% protein.

[†]Extra methionine was added to the oat diet since oat bran does not contain methionine.

[‡]Anhydrous butter contains 230 mg cholesterol/100 g. To compensate, extra cholesterol was added to the oat bran diet so that total amount of cholesterol was 0.46 g/kg diet in both diets.

[§]400 g oat bran contains 71 g protein, 206 g starch, 9 g sucrose, 65 g dietary fibre (whereof 29 g β-glucans) and 14 g ash, as analyzed by Eurofins Food Lidköping, Sweden.

[‖]Control and oat bran diets contained 18.8 and 18.7 kJ/g respectively.

Table S2. Formulation of the Western diets and macronutrient contents

Ingredient	Control	Oat bran (27%)
	g/kg diet	g/kg diet
Casein, 80 Mesh[*]	200	146
DL-Methionine[†]	3	4.5
Corn Starch	281	142
Maltodextrin	100	100
Sucrose	100	94
Cellulose	44	0
Butter, Anhydrous[‡]	200	176
Corn Oil	10	10
Mineral Mix S10026	10	10
DiCalcium Phosphate	13	13
Calcium Carbonate	5.5	5.5
Potassium Citrate, 1 H2O	16.5	16.5
Vitamin Mix V10001	10	10
Choline Bitartrate	2	2
Cholesterol[‡]	0	0.06
Oat Bran[§]	0	270
Total	1000	1000
Macronutrient contents[‖]	% energy	% energy
Protein	16	16
Carbohydrate	43	43
Fat	41	41

[*]Casein is 88% protein.

[†]Extra methionine was added to the oat diet since oat bran does not contain methionine.

[‡]Anhydrous butter contains 230 mg cholesterol/100 g. To compensate, extra cholesterol was added to the oat bran diet so that total amount of cholesterol was 0.46 g/kg diet in both diets.

[§]270 g oat bran contains 48 g protein, 139 g starch, 6 g sucrose, 44 g dietary fibre (whereof 20 g β-glucans) and 9 g ash, as analyzed by Eurofins Food Lidköping, Sweden.

[‖]Both control and oat bran diets contained 19.2 kJ/g.