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RESEARCH ARTICLE

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Differential effects of plant and animal fats on obesity-induced dyslipidemia and atherosclerosis in Ldlr−/−.Leiden mice

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Abstract

Cardiovascular disease (CVD) is closely associated with obesity through risk factors such as dyslipidemia and chronic low-grade inflammation, which may be affected by diet. Dietary fats have been extensively studied in relation to CVD risk, however these studies have not always yielded consistent results, most likely due to lack in control of experimental conditions and confounding factors. Here we studied the effects of different plant and animal fats on dyslipidemia, inflammation, and atherosclerosis. Ldlr−/−.Leiden mice were fed isocaloric energy-dense diets with translational macronutrient composition for 28 weeks. The diets were identical apart from the type of fat they contained: either (1) a mixture of olive and rapeseed oil, (2) sunflower oil, (3) pork fat, (4) beef fat, or (5) milk fat. The fatty acid composition of the diets was determined and effects on circulating lipid and inflammatory risk factors and atherosclerosis were examined, complemented by adipose tissue histology and liver transcriptomics. While visceral fat mass, adipocyte size, and adipose tissue inflammation were not differentially affected by the diets, atherosclerotic lesion load and severity was more pronounced with increasing dietary saturated fatty acid content and decreasing monounsaturated and polyunsaturated fatty acid content, and hence most pronounced with beef and milk fat. These differential effects were accompanied by increases in pro-atherogenic plasma lipids/lipoproteins (e.g., triglycerides, apolipoprotein B), activation of pro-atherogenic cytokine/chemokine signaling pathways in liver, and with circulating pro-atherogenic mediators of inflammation altogether providing a rationale for the differential effects of plant and animal fats.

Abbreviations: ApoB, apolipoprotein B; CLS, crown-like structure; CVD, cardiovascular disease; eWAT, epididymal white adipose tissue; HFD, high-fat diet; HPS, hematoxylin phloxine saffron; ICAM, intercellular adhesion molecule 1; MIF, macrophage migration inhibitory factor; MUFA, monounsaturated fatty acid; oxLDL, oxidized low-density lipoprotein; PUFA, polyunsaturated fatty acid; VCAM, vascular cell adhesion molecule 1; WAT, white adipose tissue.

Robert Kleemann and Harald Carlsen contributed equally.

KEYWORDS
adipose tissue, atherosclerosis, dietary fats, hypercholesterolemia, inflammation, macrophage migration inhibitory factor, obesity

1 | INTRODUCTION

Cardiovascular disease (CVD) is the most common cause of death in Europe,[1] despite substantial advances in prevention and treatment. There is a clear association between CVD and obesity, the prevalence of which has increased steeply over the past decades.[1,2] Obesity increases CVD risk through risk factors such as dyslipidemia[3] and chronic low-grade inflammation.[4]

Dyslipidemia in obesity is characterized by an elevation in circulating apolipoprotein B (ApoB)-containing lipoproteins, particularly those that are triglyceride-rich.[3] These ApoB-containing lipoproteins play a central causative role in atherosclerotic CVD, as the key source of cholesterol in the pathogenesis of atherosclerosis.[5,6] The trigger for atherosclerosis is formed by the subendothelial retention of plasma-derived ApoB-containing lipoproteins.[7] Oxidative modification of these lipoproteins (i.e., formation of oxidized low-density lipoproteins; oxLDL) and the subsequent chronic inflammatory response further drives the progression of atherosclerosis.[8] This inflammatory response is not just a local event within the arterial wall, but it can also be stimulated by extravascular pro-inflammatory signals that originate from other organs.[9]

One of these organs that is particularly relevant in obesity is the adipose tissue, which is thought to be an important contributor to the chronic low-grade inflammatory state that characterizes obesity. Sustained excess of energy intake leads to expansion of the adipose tissue, by hyperplasia (increase in adipocyte number) and by hypertrophy (increase in adipocyte size). Adipocyte hypertrophy compromises the functionality of the adipose tissue and ultimately leads to cell death which contributes to adipose tissue inflammation.[10,11] Dysfunctional and inflamed adipose tissue can release inflammatory mediators such as the pro-atherogenic cytokine macrophage migration inhibitory factor (MIF) into circulation, which can stimulate inflammation in distant organs including the vasculature.[12,13]

While it is well-established that genetic and epigenetic factors contribute to both obesity and CVD risk, environmental factors such as physical activity and diet also play an important role.[14] In the context of CVD, the role of dietary fats has been extensively studied and debated over several decades. It is not just the amount of dietary fat, but also the *type* of fat that determines CVD risk. Animal fats from domesticated mammals such as cattle, sheep and pigs are high in saturated fats, which are commonly assumed to increase CVD risk compared with unsaturated fats from fatty fish or plants as they tend to raise cholesterol levels more than unsaturated fats.[15] However, studies aimed at studying differences between dietary fats have not always yielded consistent results, and therefore still remain a subject of controversy.

An important factor underlying these inconsistent results is that studies attempting to replace specific fat types with other macronutrients or other fat types for comparison, often end up risking that the different diets are not properly controlled for.[16] It is crucial in such studies that the fat studied is isocalorically exchanged by an explicitly specified macronutrient. Another caveat of human studies is that most clinical studies are not performed over a sufficiently long time reflecting the slow development of CVD, which can take years or even decades to develop.

Here, we studied different dietary fat types originating from either plants (high in unsaturated fats and low in saturated fats) or animals (high in saturated fats and low in unsaturated fats). The following experimental high-fat diets were chosen: (1) a mixture of olive and rapeseed oil, (2) sunflower oil, (3) pork fat, (4) beef fat, and (5) milk fat to investigate effects on adipose tissue inflammation and atherosclerosis development over a period of 7 months. In humans this period can roughly be translated to 20 years of intervention, reflecting the difference in maturation rate between mice and humans at different ages, and also considering the maximal life span which is 3 years in mice and 120 years in man.[17,18] These fats were chosen because they are consumed world-wide and constitute the predominant dietary fats in Europe. Additionally, the different fat sources represent main classes of fatty acids rich in either monounsaturated fatty acids (MUFAs), polyunsaturated fatty acids (PUFAs), or saturated fatty acids (SFAs). While a mixture of olive- and rapeseed oil is rich in both MUFAs (olive oil) and PUFAs (n-3 and n-6; rapeseed oil), sunflower oil is predominantly a rich source of n-6 PUFAs. The selected animal fats are rich in SFAs, with appreciable but varying amounts of MUFAs and are in general low in PUFAs.

Ldlr−/−.Leiden mice, a translational model for diet-induced obesity and associated cardiovascular and metabolic diseases[19] were used for this study. These mice develop dyslipidemia with lipoprotein profiles similar to humans,[20] in conjunction with adipose tissue inflammation and vascular inflammation upon energy-dense diet feeding.[21] Herein, we used isocaloric energy-dense high-fat diets with a translational macronutrient composition relevant for humans. The diets in all groups contained 20 kcal% protein from casein, 35 kcal% of carbohydrates

from whole oat flour, and 45 kcal% of fat from the different dietary sources outlined above. The experimental diets were thus identical apart from the type of dietary fat, allowing a direct comparison of the effects of these different fats. This enabled us to evaluate circulating lipid and inflammatory risk factors that drive atherogenesis together with chronic inflammation in adipose tissue. A liver transcriptomics analysis (RNAseq) revealed pathways relevant to atherogenesis that were differentially regulated by the dietary fats, corroborating the histological quantification of atherosclerosis.

2 | MATERIALS AND METHODS

2.1 | Animal study

All animal experiments were carried out in accordance with European guidelines for the care and use of laboratory animals (European Directive 2010/63/EU) and the Norwegian national guidelines for animal welfare, and the protocol was ethically reviewed and approved by the Norwegian Food Safety Authority (ID 21501). Animal welfare was monitored by daily assessment of water and food intake, stool and fur quality, activity level, and behavior. Male Ldlr−/−.Leiden mice (genetic background 94% C57BL/6J and 6% 129S) were obtained from the breeding stock in the AAALAC-accredited SPF animal facility at TNO Metabolic Health Research. Male mice were used because they develop a more pronounced metabolic phenotype than female mice, and they are more susceptible to develop diet-induced obesity and associated diseases.[22] The animal experiment was performed in the animal facility at the Faculty of Chemistry, Biotechnology and Food Science, Norwegian University of Life Sciences (NMBU). Animals were group-housed ($n = 3$/cage) in individually ventilated cages (IVC; Innovive) in a temperature- and humidity-controlled room (24–26°C, RH: 45%–60%) on a 12-h light–dark cycle with free access to food and water. Until the start of the experiment all mice were kept on a standard rodent chow diet (Rat/Mouse Maintenance; Ssniff Spezialdïaten). At 15–18 weeks of age, mice were randomized and divided into six experimental groups. One group ($n = 9$) was kept on the chow diet as a healthy/aging reference group (chow). The other five groups were fed an energy-dense high-fat diet (HFD) that contained 45 kcal% fat from either plant or animal origin: olive and rapeseed oil (olive:rapeseed, 20:80 ratio; $n = 13$), sunflower oil ($n = 13$), pork fat ($n = 13$), beef fat ($n = 14$), or milk fat ($n = 14$). The HFDs were iso-energetic (4.75 kcal/g) and differed only in fat source. All the diets also contained soybean oil (2%–5% of the energy), with slightly different content in the various diets to adjust for total energy content. The carbohydrate source of the HFDs was fine grained whole oat flour (Norgesmøllene) and the protein source was casein (Dyets Inc.). The vitamin mix and mineral mix were based on the AIN76A vitamin mix and a DIO mineral mix (Dyets Inc.). The vitamin A in the vitamin mix added to the milk fat diet was reduced by 50% to adjust for higher vitamin A naturally present in milk fat. All diets were made in-house by mixing all the ingredients followed by manual pelleting and freeze drying. The full diet composition is provided in Table S1. All diets were vacuum packed to prohibit oxidation and stored at −20°C until use and cages were provided with fresh feed weekly.

Body weight and food intake were monitored throughout the study. Food intake data (measured weekly) is presented as the average food intake (in kcal/mouse/day) over the entire study. Blood samples for EDTA plasma isolation were collected via leg vein after a 4-h fast, in week 17 and 24 of the study. After 28 weeks, animals were euthanized after a 4-h fast by cervical dislocation under deep anesthesia with a cocktail of Zoletil Forte® (Virbac), Rompun® (Bayer), and Fentadon® (Eurovet Animal Health) (ZRF; intraperitoneally 0.01 mL ZRF/g body weight), with the following active ingredients: zolazepam (32 mg/kg), tiletamine (32 mg/kg), xylazine (4.5 mg/kg), and fentanyl (26 μg/kg). Blood for EDTA plasma isolation was collected via heart puncture and organs were isolated. Hearts with aortic roots were fixed in formalin and embedded in paraffin for atherosclerosis analysis. Livers were weighed, snap-frozen in liquid N_2, and then stored at −80°C for RNA isolation. Epididymal fat pads were weighed and fixed in formalin and embedded in paraffin for analysis of adipocyte hypertrophy and adipose tissue inflammation.

2.2 | Dietary fatty acid analysis

Diets were homogenized with a blender. The internal standard methyl tricosanoate (Methyl-C23:0) solved in dichloromethane was added to the homogenate (1.2 g of homogenate was used per diet). Samples were then methylated with 3 N methanolic HCl at 80°C for 2 h and then at 50°C overnight. FAMES were extracted to hexane and samples were then neutralized with 3 N KOH in water. After vortex and centrifugation, the organic phase was analyzed by gas chromatography. Analysis was performed on a 8890 GC system with a split/splitless injector, a 7693A automatic liquid sampler, with a flame ionization detector (Agilent Technologies). Separations were performed on a Varian CP7421 (200 m × 0.25 mm i.d.) column from Varian Inc. The fatty acids were identified using a fatty acid standard mixture (37 FAME-mix, Supelco, and 1269119 USP FAME standard mixture, Sigma-Aldrich) and the total amount of fatty acids were quantified.

2.3 | Dietary and fecal cholesterol analysis

In week 18 of the study, feces were collected from each cage over a period of 7 days and food intake was also determined during the same period. Dietary cholesterol content and fecal cholesterol content (reflected by the sum of the neutral sterols coprostanol, cholesterol, cholestanol, and lathosterol) were determined by gas chromatography as described previously.[23,24] Dietary/fecal samples were lyophilized and weighed. Samples were then incubated in 1 mL alkaline methanol (3:1 v/v) for 2 h at 80°C, using 5α-cholestane as an internal standard. Next, neutral sterols were extracted three times with petroleum ether. The combined petroleum layers were evaporated after which the neutral sterols were silylated with DMF Sil-prep. Next they were separated by gas chromatography using a 25 m × 0.25 mm capillary gas chromatography column (CP-Sil 5B, Agilent; temperature programmed from 230 to 280°C) in a Scion 436-GC gas chromatography system (Scion Instruments) equipped with a flame ionization detector (kept at 300°C). Neutral sterol derivatives were introduced by split injection (split ratio, 20:1; injector temp 300°C). Quantitation of neutral sterols was based on the area ratio of the individual neutral sterol to the internal standard. These data were combined with the food intake measurements or the fecal excretion measurements to calculate the dietary cholesterol intake and the fecal neutral sterol excretion (both expressed as μmol/mouse/day).

2.4 | Plasma analyses

Plasma total cholesterol and triglycerides were determined using the enzymatic assays Cholesterol Gen.2 and TRIGL respectively (both Roche Diagnostics) according to the manufacturer's instructions. Commercially available ELISA kits were downscaled[25,26] and then used according to the manufacturer's instructions for the analysis of plasma oxLDL (Cusabio), ApoB100 (Abcam), MIF, E-selectin, P-selectin, ICAM-1, and VCAM-1 (all R&D Systems).

2.5 | Adipose tissue analysis

Paraffin-embedded epididymal white adipose tissues (WAT) were sectioned (5 μm) and then stained with hematoxylin and eosin (Tissue-Tek H&E staining kit, Sakura). Slides were digitized with a slide scanner (NanoZoomer S210 Digital slide Scanner, Hamamatsu). Images were exported using NPD.view 2 software (Hamamatsu) and subsequently analyzed for morphometry (cell size and number) using Adiposoft,[27] an open-source automated plug-in for the image processing package Fiji[28] for ImageJ.[29] In addition, inflammation was analyzed in each image as previously described[30] by counting the number of crown-like structures (CLS; a histological hallmark of adipose tissue inflammation[31]), which was expressed per 1000 adipocytes. The average tissue area examined was 2.2 mm^2.

2.6 | Atherosclerosis analysis

Paraffin-embedded hearts with aortic roots were sectioned perpendicular to the axis of the aorta and then stained with hematoxylin phloxine saffron (HPS). Per mouse, four serial cross-sections (5 μm) at 50-μm intervals were analyzed, with experimental groups blinded to the assessor. Slides were digitized with a slide scanner (Aperio AT2, Leica Biosystems) for morphometric analysis of lesion number and area and analysis of lesion severity (using ImageScope v12.3.2.8013 software, Leica Biosystems) according to the classification of the American Heart Association.[32,33] This scoring system was used to distinguish five lesion types: (I) early fatty streak with up to 10 foam cells in the intima, no other changes; (II) regular fatty streak with 10 or more foam cells in the intima, no other changes; (III) mild plaque with foam cells in the intima and presence of a fibrotic cap; (IV) moderate plaque, progressive lesion with infiltration into media, elastic fibers intact; and (V) severe plaque, structure of media severely disrupted with fragmented elastic fibers, cholesterol crystals, calcium deposits, and necrosis may be present.

2.7 | Liver transcriptomics analysis

Total RNA was extracted from liver samples RNA-Bee (Campro Scientific) and purified using PureLink RNA Mini Kit (Thermo Fisher Scientific) as previously described.[34] Next generation sequencing was performed by GenomeScan B.V. Sample preparation was performed using NEBNext Ultra II Directional RNA Library Prep Kit for Illumina (#E7760S/L, New England Biolabs) according to the manufacturer's instructions. In brief, mRNA was extracted from total RNA using oligo-dT magnetic beads. After fragmentation of the mRNA, cDNA was synthesized followed by ligation with the sequencing adapters and amplification of the resulting product by PCR. Quality and yield of the amplicon was determined (Fragment Analyzer, Agilent Technologies) and fulfilled QC-criteria (broad peak between 300 and 500 bp). Clustering and DNA sequencing, using the NovaSeq6000 (Illumina), was performed according to manufacturer's protocols, yielding 14–35 million sequencing clusters per sample and 2 × 150 bp paired-end reads (PE) per cluster. Data were processed as described previously.[35] Differentially expressed genes (DEGs; *p*-value

TABLE 1 Fatty acid composition and cholesterol content of the experimental diets.

Test product	SCFA C4:0-C8:0	C10:0	Sum SFA[a]	Sum MUFA	Sum C18:1t	Sum CLA	Sum PUFA	n6:n3 ratio	Cholesterol
Chow	<LOQ	<LOQ	0.69 ± 0.001^{a}	0.64 ± 0.02^{a}	<LOQ	<LOQ	2.00 ± 0.004^{a}	9.01 ± 0.02^{a}	4.0 ± 0.3^{a}
Olive/rapeseed oil	<LOQ	<LOQ	2.41 ± 0.01^{b}	13.61 ± 0.08^{b}	<LOQ	<LOQ	7.32 ± 0.02^{b}	3.94 ± 0.01^{b}	8.3 ± 0.4^{ab}
Sunflower oil	<LOQ	<LOQ	3.05 ± 0.03^{c}	7.21 ± 10^{c}	<LOQ	<LOQ	12.73 ± 0.15^{c}	81.11 ± 1.06^{c}	11.3 ± 0.4^{bc}
Pork fat	<LOQ	<LOQ	8.23 ± 0.11^{d}	11.04 ± 0.11^{d}	0.02 ± 0.01^{a}	0.013 ± 0.007^{a}	5.65 ± 0.06^{d}	10.05 ± 0.23^{b}	15.5 ± 0.6^{c}
Beef fat	<LOQ	0.02 ± 0.000^{a}	10.42 ± 0.02^{e}	10.18 ± 0.01^{e}	0.32 ± 0.01^{b}	0.043 ± 0.005^{b}	3.26 ± 0.02^{e}	9.30 ± 0.53^{b}	27.0 ± 2.0^{d}
Milk fat	1.30 ± 0.05	0.62 ± 0.002^{b}	15 ± 0.04^{f}	5.81 ± 0.05^{f}	0.32 ± 0.02^{b}	0.034 ± 0.002^{ab}	2.33 ± 0.01^{a}	9.65 ± 0.04^{b}	54.6 ± 4.5^{e}

Note: Amount of fatty acid (FAME) g/100 g total diet, ratio between n6 and n3 fatty acids, and cholesterol content (mg/100 g diet). Average total fat in the HFDs was 24 g/100 g, chow had 3.5 g fat/100 g. N.B. data reflect fatty acid composition and n6:n3 ratio of total diet, which also includes a small amount of fat from other ingredients, for example, the carbohydrate source oat flour, and may therefore differ from what would be expected from the respective pure fats. Data shown are mean ± SEM, groups with corresponding superscript letters are statistically comparable ($p > .05$).

Abbreviations: CLA, conjugated linoleic acid; LOQ, limit of quantitation; MUFA, mono-unsaturated fatty acid; PUFA, poly-unsaturated fatty acid; SCFA, short-chain fatty acid; SFA, saturated fatty acid.

[a]Short-chain fatty acids were included.

cut off for differential expression: $p < .003$) were determined with the DESeq2 method[36] and these DEGs served as the input for analysis with Ingenuity Pathway Analysis Suite.[37] This analysis uses gene expression patterns in predefined biological pathways or downstream from upstream regulators (e.g., cytokines, transcription factors, metabolites) to predict their activation state as previously described.[24] Significance of enrichment of an upstream regulator or biological pathway is indicated by the p-value ($-\log p > 2$ was considered statistically significant). A Z-score indicates the enhanced or reduced activity of an upstream regulator or pathway ($Z > 2$ = predicted activation, $Z < -2$ = predicted reduction in activity). Analyses were performed for the comparison of each energy-dense HFD with chow, and for each energy-dense HFD with the olive/rapeseed oil group.

2.8 | Statistics

With the exception of the transcriptomics analysis, for which the statistical analyses have been described above, the statistical analysis was performed using Graphpad Prism version 8.0.1 (244) for Windows (Graphpad Software). Normal distribution of variables was assessed using the Shapiro–Wilk normality test ($\alpha = .05$). Homogeneity of variances was assessed using the Brown–Forsythe test ($\alpha = .05$). For normally distributed variables with equal variances, differences between groups were analyzed by one-way analysis of variance (ANOVA) followed by Tukey's post-hoc tests. For normally distributed variables with unequal variances, differences between groups were analyzed by analysis of variance (Brown–Forsythe) and Tamhane's T2 post-hoc tests. For variables that were not normally distributed, the non-parametric Kruskall–Wallis test followed by post-hoc with Dunn's tests were used to determine differences between groups. $p < .05$ were considered statistically significant. Data are represented as mean ± SD.

3 | RESULTS

3.1 | Fatty acid composition and cholesterol content of the experimental diets

Prior to the start of the study, we characterized the fatty acid composition and cholesterol content of the experimental diets (an overview is provided in Table 1, the full fatty acid composition of each diet is provided in Table S2). Overall, the chow diet (which was included as a non-obese reference) was lowest in fat (9% by energy) and was correspondingly also lowest in the different fatty acid types. Between the energy-dense HFDs—which all contained the same amount of dietary fat (45% by

energy)—the plant-derived fat diets (olive/rapeseed oil and sunflower oil) had the lowest SFA content, and SFA were considerably higher in the animal-fat diets (pork fat, beef fat, milk fat), especially in the milk fat group. With regard to MUFAs and PUFAs, both pork and beef fat-based diets had appreciable amounts of MUFAs with levels matching the plant-derived diets. Milk fat had the lowest MUFA content. PUFAs were clearly higher in the plant diet groups compared with the animal fats, but pork fat was substantially higher in PUFAs than both beef and milk fat. The two plant-derived oil diets differed in their n6:n3 ratio, which was low for the olive/rapeseed oil diet and high for the sunflower oil diet. The animal fat diets were characterized by small amounts of short-chain fatty acids (specifically in the milk fat diet) and medium-chain fatty acids (decanoic acid C10:0 in beef and milk fat diets), as well as the trans fatty acid vaccenic acid (C18:1t) and conjugated linoleic acid (all animal fat diets), all of which were undetectable in the plant-fat diets. Dietary cholesterol was lowest in the chow diet, slightly higher in the plant oil diets and more pronouncedly increased in the animal fat diets—especially the beef and the milk fat diets.

3.2 | Dietary fat types differentially affect body weight but not adipose tissue inflammation

At baseline, the average body weight in all groups was comparable (28.3 ± 5.5 g). All energy-dense HFDs significantly increased body weight relative to the chow-fed controls (Figure 1A). The weight gain caused by the HFDs was numerically least pronounced for the olive/rapeseed oil diet, intermediate for the sunflower oil diet and most pronounced for the three animal fat diets. Differences in body weight increments were not explained by food intake, which was comparable between the HFDs (Figure 1B).

In line with the effects on body weight, all energy-dense HFDs significantly increased epididymal WAT (eWAT) mass relative to chow-fed controls (Figure 1C). The increase in the HFD groups was statistically comparable between the HFDs. This increase in eWAT mass by all HFDs was accompanied by a significant increase in average adipocyte size (hypertrophy) relative to chow (Figure 1D), with no difference between the HFDs. Similarly, all energy-dense HFDs increased eWAT inflammation relative to chow with no differences between the energy-dense diets and considerable within-group variation (Figure 1E, and representative photomicrographs in Figure S1).

3.3 | Plasma lipids are most strongly increased by the animal-fat diets

Plasma lipids were significantly increased relative to chow-fed controls by all of the energy-dense HFDs (Figure 2A,B). In week 24, plasma triglycerides (Figure 2A) were low in chow-fed controls. The plant oil diets modestly increased plasma triglycerides to a comparable extent. The animal fats all increased plasma triglycerides more strongly than the plant oils. This effect was least pronounced in the pork fat group, intermediate in the beef fat group and most strong in milk fat dairy group. A similar pattern was observed for plasma cholesterol levels (Figure 2B). In

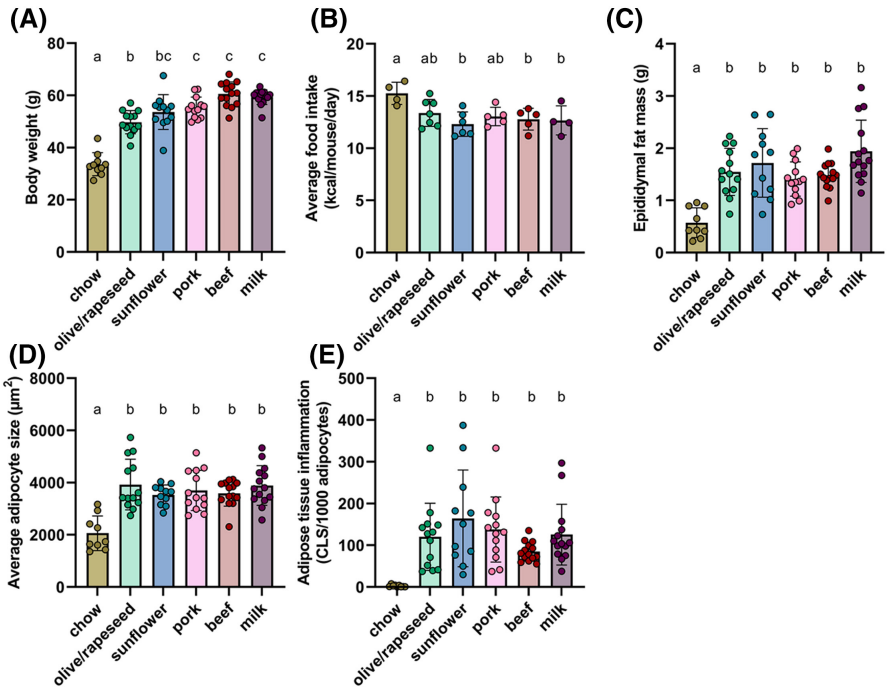

FIGURE 1 Energy-dense high-fat diets (HFDs) increase body weight and adiposity. Ldlr−/−.Leiden mice were fed energy-dense HFDs that were identical apart from their fat source for 28 weeks. (A) Effect of HFD feeding on body weight in week 28 of the study. (B) Average food intake during the study was comparable on all HFDs. (C) Fat pad mass, (D) average adipocyte size, and (E) inflammation in the epididymal adipose tissue were comparably increased by all HFDs. CLS, crown-like structure. Data shown are mean ± SD. Bars with corresponding letters are statistically comparable ($p > .05$).

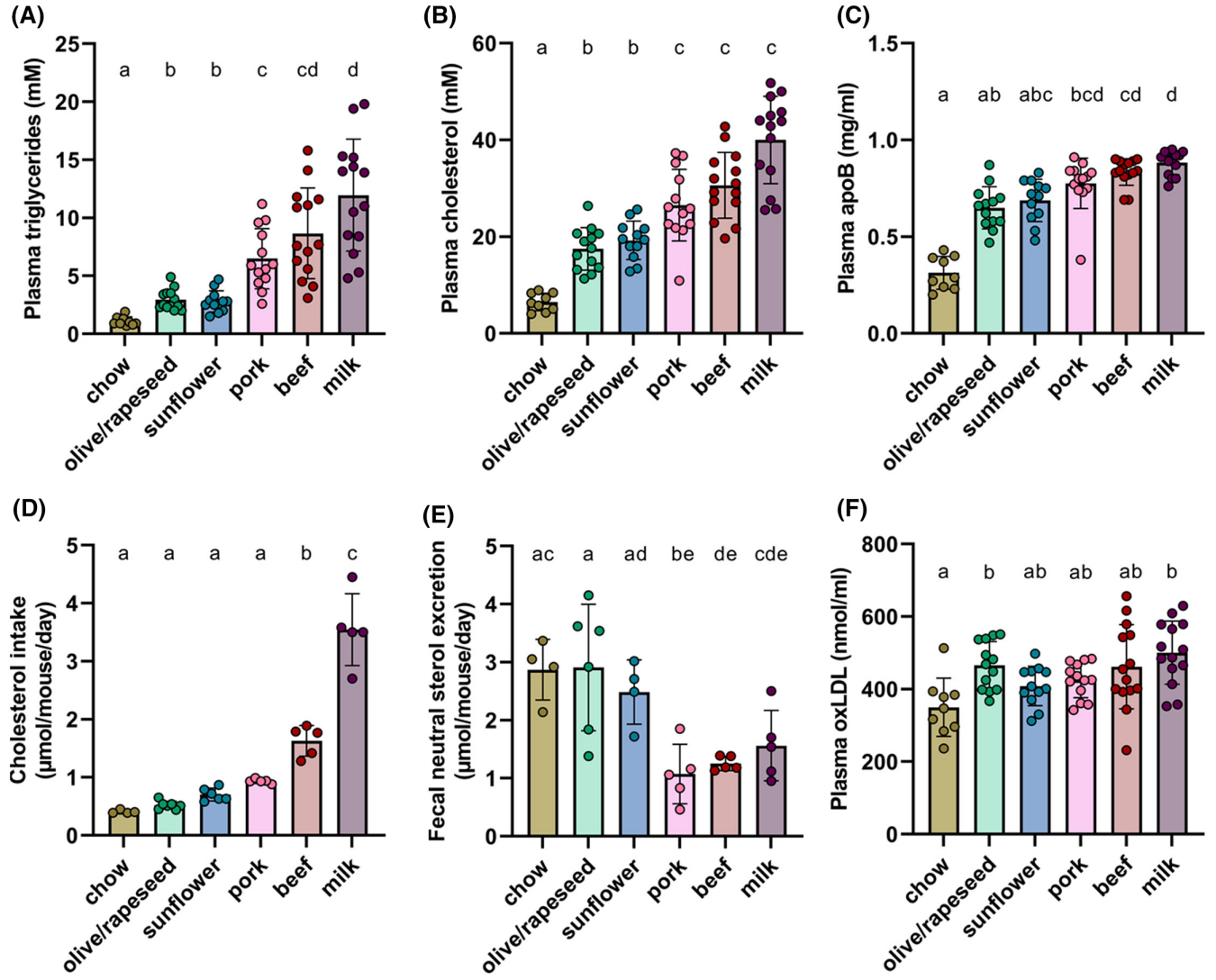

FIGURE 2 Energy-dense high-fat diets (HFDs) increase plasma lipids. Ldlr−/−.Leiden mice were fed energy-dense HFDs that were identical apart from their fat source for 28 weeks. Fasting (4 h) plasma triglycerides (A), total cholesterol (B), ApoB-100 (C) in week 24 of the study. (D) Dietary cholesterol intake and E) fecal neutral sterol excretion in week 18 of the study. (F) Fasting (4 h) oxidized LDL (oxLDL; D) in week 24 of the study. Data shown are mean ± SD. Bars with corresponding letters are statistically comparable ($p > .05$).

chow-fed controls they were low. The plant-derived oils increased circulating cholesterol to a similar extent. The three animal-derived fats more strongly elevated total plasma cholesterol levels, to a comparable degree.

This increase in total cholesterol levels was paralleled by an increase in plasma ApoB, indicative of higher atherogenic non-HDL lipoprotein particle concentrations,[38] specifically in the animal-fat diets (Figure 2C). In chow-fed animals, plasma ApoB was low. Plasma levels in the plant oil diets were statistically comparable to chow, while all animal-fat diets significantly increased plasma ApoB—an effect that was most pronounced for the milk fat group. To investigate whether these changes in plasma cholesterol and plasma ApoB levels may be related to differences in cholesterol intake and excretion, we assessed dietary cholesterol intake and fecal cholesterol loss in week 18 of the study. While cholesterol intake was comparable to chow in the olive/rapeseed oil-, sunflower oil- and pork fat groups, it was significantly increased in the beef fat group and even more so in the milk fat group (Figure 2D). Fecal cholesterol excretion on the other hand was statistically comparable to chow in the two plant-fat groups and the milk fat group. Fecal cholesterol excretion was significantly lower than chow in the pork fat and the beef fat group (Figure 2E).

Only the olive/rapeseed oil diet and the milk-fat diet significantly increased plasma oxLDL—a strong predictor of CVD in humans[39]—(Figure 2F) relative to chow. The sunflower oil group, the pork fat group and the beef fat group had intermediate levels of oxLDL, which where statistically comparable to both the chow controls and to the olive/rapeseed oil and milk fat groups.

3.4 | Atherosclerosis development is more pronounced (in both area and severity) in animal-fat diets

Finally, we analyzed atherosclerosis development in the aortic root (Figure 3A and representative images provided in Figure S2). All HFDs increased the total lesion area significantly relative to chow. This effect was least pronounced in the plant-fat diets. The pork-fat diet had an intermediate effect and the beef- and milk-fat diets most strongly induced atherosclerotic lesion load.

A refined analysis of lesion severity showed that all energy-dense HFDs resulted in the development of severe atherosclerosis. The mild type I and type II lesions made up less than 1% of the total lesion area in all groups (not shown). In the olive/rapeseed oil group, the relative contribution of type III lesion area (Figure 3B) to the total lesion load was comparable to chow. Sunflower oil, pork fat and beef fat had a numerically but not statistically lower percentage of type III lesion area and the milk fat diet resulted in a significantly reduced relative type III lesion area. While the plant oil diets did not significantly affect the percentage of type IV lesion area (Figure 3C) relative to chow, all animal fats significantly lowered the relative type IV lesion area to a comparable extent. This reduction in the relative area of type IV lesions was accompanied by an increase in type V lesion area (Figure 3D), the most severe lesion type (a shift that is indicative of lesion progression). The olive/rapeseed oil group had a numerically increased but statistically comparable percentage of type V lesion area relative to chow. All other energy-dense diets significantly increased the type V lesion area relative to chow. This effect was least pronounced in the sunflower oil group, intermediate in the pork fat and beef fat groups, and most prominent in the milk fat group. Altogether these data indicate that all HFD-fed groups developed

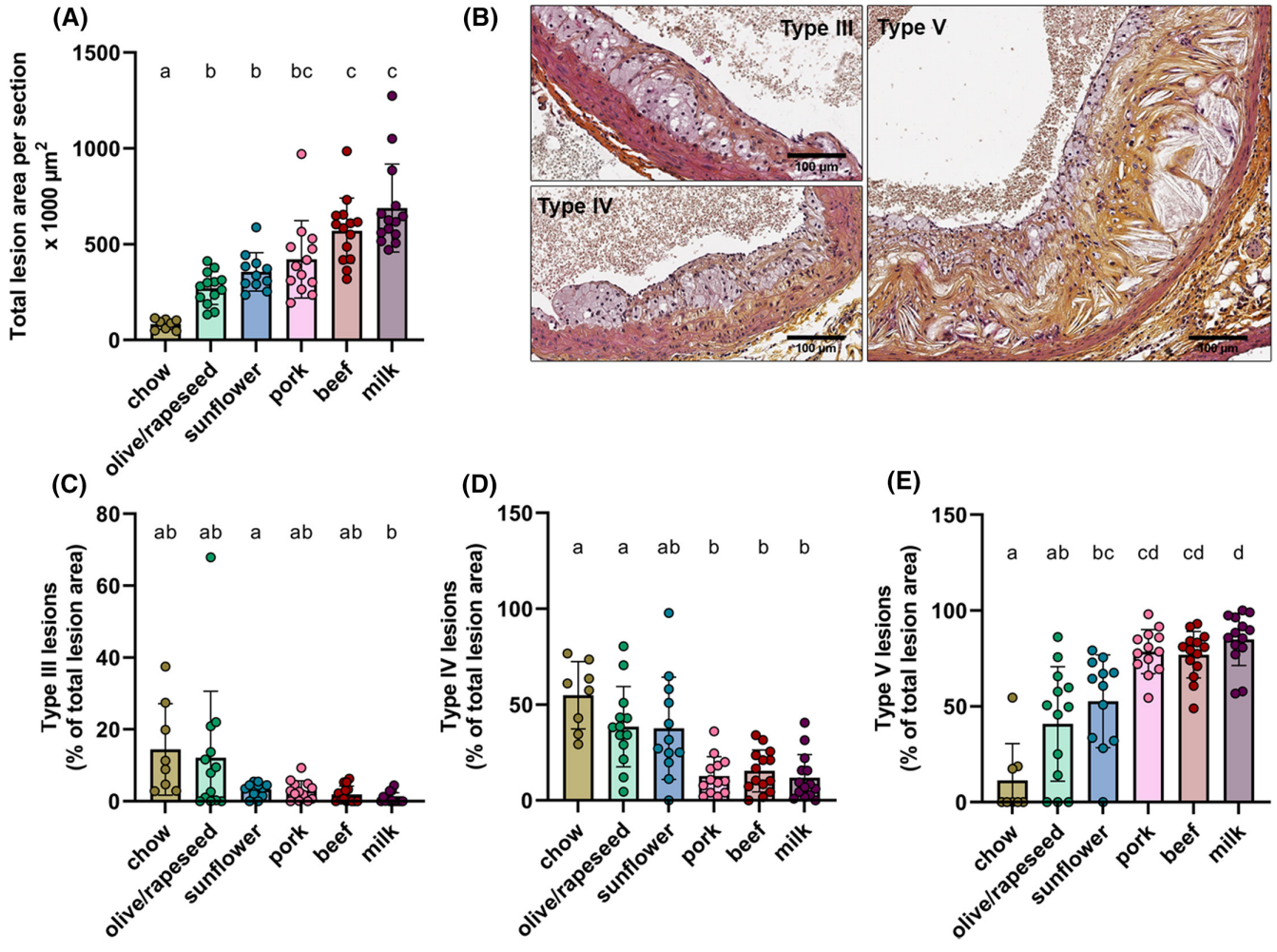

FIGURE 3 Effects of energy-dense high-fat diets (HFDs) on atherosclerotic lesion development in the aortic root. Ldlr−/−.Leiden mice were fed energy-dense HFDs that were identical apart from their fat source for 28 weeks. (A) total atherosclerotic lesion area. (B) Representative images of a type III, type IV and a type V lesion. (C)percentage of type III (mild) lesions. (D) percentage of type IV (moderate) lesions. (E) percentage of type V (severe) lesions. Data shown are mean ± SD. Bars with corresponding letters are statistically comparable ($p > .05$).

severe atherosclerosis—as is observable from the large contribution (>50%) of the severe type IV and type V lesion types to the total lesion area. Lesion development was more severe in the animal-fat diets than in the plant-fat diets, as is apparent from the shift from type IV lesion area (which was reduced) toward type V lesion area (which was increased).

3.5 | Effects of energy-dense high-fat diets on atherosclerosis-related gene expression

To further investigate the mechanisms underlying the differences between the energy-dense diets with different fat sources, we next studied biological processes based on hepatic transcriptomics analysis (RNAseq). In this analysis, we used downstream gene expression patterns to predict the activation status of biological pathways and upstream regulators. We focused firstly on the processes that were affected by the energy-dense diets relative to chow. Next, we studied the differences between the HFDs by comparing each group with the olive/rapeseed oil group (the HFD that numerically had the lowest total atherosclerotic lesion load) to better allow detection of differences between the energy-dense HFDs.

As expected, all of the energy-dense HFDs modulated pathways relevant to atherosclerosis; including pathways involved in lipid metabolism, inflammation, endothelial (dys)function, vasoregulation, and thrombogenesis. An overview of all significantly regulated pathways is provided in Table S3. For brevity, we focused on pathways that were differentially regulated between the plant oil diets and the animal fat diets. The canonical pathway "*Atherosclerosis Signaling*" was significantly enriched by all HFDs relative to chow-fed controls. This effect was most pronounced for the animal fat diets—which all differed significantly from the olive/rapeseed oil group—while the two plant oil groups were comparable. Conversely, the atheroprotective signaling pathways "*LXR/RXR activation*" and "*PPAR Signaling*"[40,41] were inactivated by all HFDs relative to chow, with more pronounced inactivation in the animal fat groups relative to olive/rapeseed oil while the two plant oil groups were comparable. The thrombogenic pathway "*GP6 signaling*"[42] was activated by all energy-dense HFDs, with more prominent activation by the beef and milk fat groups relative to olive/rapeseed oil. Furthermore, all HFDs increased activation of "*Renin–Angiotensin Signaling*," the "*Apelin Endothelial Signaling Pathway*," and "*Thrombin Signaling*"—three proatherogenic pathways involved in vasoregulation, endothelial function and inflammation, and thrombogenesis.[43–45] The milk fat diet significantly increased the activation of these pathways relative to olive/rapeseed oil, while the other HFDs were comparable.

We then studied the effects of the energy-dense diets on the predicted activation state of upstream regulators involved in cholesterol metabolism and handling (Table 2) and of upstream regulators involved in inflammation (Table 3). An overview of all significantly regulated upstream regulators is provided in Table S4. The olive/rapeseed oil diet tended to increase acgenes that promote cholesterol synthesis, as indicated by increased activation of SREBF2 and inactivation of INSIG1 (a negative regulator of cholesterol synthesis). The other HFDs did not have a clear effect on SREBF2 activation relative to chow, but did show inactivation of INSIG1, pointing to an overall increase in cholesterol synthesis. This was also reflected by the comparison of the other HFDs. Relative to the olive/rapeseed group all the other HFDs showed inactivation of SREBF2 and SREBF2 chaperone protein SCAP, and activation of INSIG1.

None of the HFDs had a clear effect on regulators related to VLDL production and secretion. Similarly, there was no clear pattern of activation of (V)LDL receptors and associated genes. LRP1 (a cholesterol clearance receptor) tended to be inactivated and PCSK9 (which promotes lysosomal Ldlr degradation) tended to be activated by the energy-dense diets, but these effects did not reach the cut off for consistent (in)activation for any of the diets. There was a significant inactivation of ApoE by all HFDs, which tended to be more pronounced for the animal fat diets. The chaperone protein LRPAP1 tended to be more activated in all HFDs relative to chow, and this increased activation was most prominent in the animal fat diets. HDL-related genes overall were downregulated by the HFDs relative to chow, with no clear differences relative to the olive/rapeseed diet. All HFDs inactivated the atheroprotective bile acid regulator FXR relative to chow, and FXR was further inactivated only in the milk fat group relative to olive/rapeseed. There was no clear effect of the HFDs on bile acid synthesis (CYP7A1 and CYP27A1) or the efflux of cholesterol into bile (ABCA1).

The energy-dense HFDs resulted in the activation of many pro-atherogenic chemokines and/or their receptors, such as CCR2, CCL5, CXCL12, CX3CL1, and MIF (Table 3). The sunflower oil was comparable to olive/rapeseed oil with respect to chemokine signaling, while the animal fats further increased CCR2 (all animal fats), CCL5 (beef fat and milk fat), CCR1 (milk fat), CXCL12 (beef fat and milk fat), CX3CL1 (all animal fats), CX3CR1 (milk fat), and MIF (beef fat and milk fat) activity. Similarly, there was a global activation of pro-inflammatory pro-atherogenic cytokines by all HFDs, including IL1B, IL3, IL6, CSF1, and TNF. The activation of these cytokines was comparable between the two plant fat diets (with the exception of IL1B, which

TABLE 2 Cholesterol-related upstream regulators.

	Olive/rapeseed oil		Sunflower oil		Pork fat		Beef fat		Milk fat		Sunflower oil		Pork fat		Beef fat		Milk fat	
	Chow		Chow		Chow		Chow		Chow		Olive/rapeseed oil		Olive/rapeseed oil		Olive/rapeseed oil		Olive/rapeseed oil	
Comparison	*Z*-score	−logP	*Z*-score	−logP	*Z*-score	−logP	*Z*-score	−logP	*Z*-score	−logP	*Z*-score	−logP	*Z*-score	−logP	*Z*-score	−logP	*Z*-score	−logP
Cholesterol synthesis																		
SREBF2	2.1	5.1	0.5	3.7	−1.2	12.1	−0.4	9.8	−0.5	11.5	−2.9	5.9	−5.2	22.6	−4.6	11.3	−3.8	13.4
SCAP	1.6	5.5	−0.9	4.4	−2.0	13.5	−1.4	11.0	−1.6	12.0	−3.1	7.6	−5.4	25.2	−4.4	14.5	−4.6	17.7
INSIG1	−4.6	12.2	−3.4	7.2	−2.1	10.2	−3.0	9.5	−2.9	9.7	2.7	7.8	4.0	14.7	2.2	7.6	1.2	12.1
HMGCR	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	1.5	0.0	0.0	0.0	0.0
VLDL production and secretion																		
MTTP	−1.0	2.6	−1.3	3.0	0.0	0.0	−1.2	2.1	−1.9	4.1	0.0	2.0	0.0	0.0	−1.6	1.8	−1.9	2.2
APOB	0.0	0.0	0.0	0.0	0.0	1.7	0.0	2.0	0.0	0.0	0.0	4.0	0.0	0.0	0.0	0.0	0.0	0.0
SORT1	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
MAT1A	2.0	4.0	0.6	3.3	1.1	4.1	1.6	3.4	0.9	4.8	0.0	0.0	0.8	5.3	1.5	1.7	0.2	2.2
(V)LDL receptors and associated genes																		
VLDLR	0.9	1.4	0.0	0.0	0.0	0.0	0.0	0.0	0.2	2.5	0.0	0.0	0.0	0.0	0.0	0.0	−0.1	2.0
LDLR	0.8	23.5	−0.3	24.4	0.5	23.1	0.3	25.1	0.4	28.0	−0.2	2.8	0.1	4.4	0.6	5.4	0.6	9.3
LRP1	−1.6	4.7	−1.7	5.3	−1.4	2.2	−1.5	2.6	−2.1	3.9	0.0	0.0	−0.5	3.4	−0.7	6.0	−0.9	7.4
PCSK9	0.8	1.4	1.2	1.6	1.5	2.4	1.2	2.0	0.0	0.0	0.0	0.0	1.9	2.7	0.9	2.2	1.0	2.1
APOE	−2.6	19.8	−2.6	16.6	−2.6	18.7	−2.6	19.6	−2.7	21.0	0.0	2.2	−0.9	5.9	−1.2	12.9	−1.9	18.5
LRPAP1	0.8	5.6	2.2	3.2	1.6	2.5	1.1	2.5	1.7	3.7	0.0	0.0	1.4	2.8	2.6	2.6	2.3	4.0
HDL-related genes																		
SCARB1	0.0	0.0	−2.1	1.5	−2.1	0.7	−2.7	1.1	−2.2	1.5	0.6	3.3	0.3	2.1	−0.9	2.9	−1.2	4.5
APOA1	−2.5	3.2	−2.5	2.3	−2.5	2.6	−1.8	1.5	−1.9	2.4	1.0	2.1	0.8	1.8	−0.5	3.8	−0.8	4.3
ABCA1	−0.9	3.8	−0.8	2.0	−0.2	2.0	−0.2	1.7	−1.2	3.2	0.0	1.6	0.9	2.8	0.6	2.4	0.6	2.3
Bile acid synthesis and cholesterol efflux																		
NR1H4	−3.3	15.3	−3.4	11.2	−2.5	6.8	−2.5	6.6	−3.2	9.1	0.9	2.2	−0.8	5.4	−1.3	3.8	−2.2	6.7
Cyp7A1	1.2	2.1	0.7	2.2	0.9	1.6	1.6	2.7	0.9	2.6	−1.3	4.8	−1.4	2.9	0.0	3.4	0.2	4.1
Cyp27A1	0.0	0.0	0.0	0.0	0.0	0.0	−0.9	2.0	−1.9	1.0	−0.3	3.7	0.0	1.4	−1.6	2.2	−1.2	3.6
ABCA1	−0.9	3.8	−0.8	2.0	−0.2	2.0	−0.2	1.7	−1.2	3.2	0.0	1.6	0.9	2.8	0.6	2.4	0.6	2.3

Note: The predicted activation state of upstream regulators is shown for the comparison of each HFD relative to chow (left half of table) and for the sunflower oil, pork fat, beef fat, and milk fat diets relative to olive/rapeseed oil (right half of table). Regulators with a −log(*p*-value) > 2 are considered to be significantly enriched. The *Z*-score indicates predicted activation (cutoff >2.0, marked in red) or inactivation (cutoff <−2, marked in green) of a regulator.

TABLE 3 Inflammation-related upstream regulators.

	Olive/rapeseed oil		Sunflower oil		Pork fat		Beef fat		Milk fat		Sunflower oil		Pork fat		Beef fat		Milk fat	
	Chow		Chow		Chow		Chow		Chow		Olive/rapeseed oil		Olive/rapeseed oil		Olive/rapeseed oil		Olive/rapeseed oil	
Comparison	*Z*-score	−logP	*Z*-score	−logP	*Z*-score	−logP	*Z*-score	−logP	*Z*-score	−logP	*Z*-score	−logP	*Z*-score	−logP	*Z*-score	−logP	*Z*-score	−logP
Pro-atherogenic chemokines and their receptors																		
CCL2	1.8	6.6	1.7	4.9	2.4	4.4	2.1	4.6	1.9	6.1	1.4	2.0	0.8	1.7	0.0	0.0	0.5	2.4
CCR2	5.0	14.4	5.2	14.6	5.5	11.7	5.5	12.5	5.2	14.7	0.0	0.0	2.2	4.2	3.8	8.5	5.1	12.8
CCL5	1.5	3.7	2.5	3.0	2.6	3.3	2.2	4.5	2.5	6.2	0.0	0.0	1.0	2.2	2.0	2.1	2.9	3.2
CCR1	1.4	4.2	1.4	3.4	1.9	2.6	1.6	2.5	1.9	3.6	0.0	0.0	0.0	0.0	0.0	0.0	2.0	4.3
CXCL1	0.0	0.0	1.6	2.3	0.0	0.0	0.0	0.0	1.6	1.5	0.0	0.0	0.0	0.0	0.0	0.0	2.0	1.9
CXCL2	2.2	1.4	2.4	1.6	2.2	0.8	2.2	0.6	2.6	1.4	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
CXCR2	0.3	1.7	0.5	1.8	0.0	1.3	0.0	0.0	0.7	1.3	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
CXCL5	2.2	2.3	2.0	1.3	2.2	1.6	2.2	1.4	2.4	2.0	0.0	0.0	0.0	0.0	0.0	1.4	0.0	1.4
CXCL12	2.4	7.6	3.1	5.3	3.0	5.9	1.9	7.8	2.9	7.0	0.0	0.0	0.0	0.0	2.5	2.8	3.4	4.9
CXCR2	0.3	1.7	0.5	1.8	0.0	1.3	0.0	0.0	0.7	1.3	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
CXCR4	0.8	1.6	0.0	0.0	2.1	1.8	1.5	2.0	2.0	2.2	0.0	0.0	0.4	1.6	1.4	2.4	1.7	2.8
CX3CL1	2.4	7.6	2.8	6.6	2.7	8.4	2.5	6.9	2.2	8.3	0.0	0.0	2.0	3.9	1.6	6.4	2.5	5.6
CX3CR1	0.9	1.7	0.7	2.0	1.7	1.4	0.0	0.0	1.2	1.4	0.0	0.0	2.1	1.0	2.9	0.6	2.9	2.3
MIF	1.2	2.6	1.5	1.8	1.6	1.6	2.1	2.4	2.5	3.1	0.0	0.0	0.0	0.0	2.8	2.9	1.7	3.1
Pro-atherogenic cytokines																		
IL1A	3.7	11.2	4.6	10.7	5.4	7.4	5.4	8.4	5.5	11.2	0.0	0.0	0.0	0.0	3.5	4.0	4.7	8.9
IL1B	3.4	36.3	4.8	36.6	5.2	35.1	5.5	33.0	5.1	35.3	2.3	2.6	2.7	5.5	3.8	14.9	4.9	27.6
IL2	2.0	11.5	2.6	15.4	3.1	15.4	4.1	16.8	4.0	16.4	1.9	0.3	0.0	0.0	1.1	2.7	2.6	3.5
IL3	2.0	13.4	2.3	14.5	2.0	18.6	2.6	18.4	3.4	18.4	0.0	0.0	0.4	3.2	1.4	8.6	2.6	5.8
IL5	2.7	10.9	3.0	8.1	2.9	13.9	2.9	10.6	3.9	10.8	0.0	0.0	−1.0	2.7	−0.9	3.0	1.7	4.5
IL6	1.0	32.9	2.1	32.9	2.6	30.4	3.4	23.9	2.5	28.1	0.0	0.0	1.8	4.6	2.9	9.8	3.5	15.6
IL13	3.8	21.3	3.0	22.6	3.4	19.9	2.8	19.9	3.4	23.6	−0.3	1.4	−0.3	6.7	0.9	13.0	1.6	17.2
IL15	2.2	11.3	2.9	8.9	3.0	9.3	3.5	7.9	3.8	10.6	0.0	0.0	0.0	0.0	0.5	2.0	0.5	2.3
IL18	3.2	0.9	3.2	1.5	3.4	1.2	3.0	1.0	3.5	1.6	0.0	0.0	0.0	0.0	0.0	0.0	1.9	1.8
GDF15	1.8	2.5	1.5	3.3	1.8	2.1	1.7	2.7	1.3	3.3	0.0	0.0	0.0	0.0	0.0	1.7	0.0	1.6
CSF1	3.9	24.7	3.7	21.9	3.5	24.8	4.0	25.5	4.4	29.5	0.0	0.0	−1.2	16.3	1.3	17.3	2.0	27.1
CSF2	4.8	26.3	5.5	25.9	6.2	26.7	6.6	26.4	7.1	28.1	0.2	2.2	2.5	2.0	3.6	7.9	5.1	10.3

(Continues)

TABLE 3 (Continued)

Comparison	Olive/rapeseed oil		Sunflower oil		Pork fat		Beef fat		Milk fat		Sunflower oil		Pork fat		Beef fat		Milk fat	
	Chow		Chow		Chow		Chow		Chow		Olive/rapeseed oil		Olive/rapeseed oil		Olive/rapeseed oil		Olive/rapeseed oil	
	Z-score	−logP	Z-score	−logP	Z-score	−logP	Z-score	−logP	Z-score	−logP	Z-score	−logP	Z-score	−logP	Z-score	−logP	Z-score	−logP
CSF3	1.8	10.5	1.4	9.7	1.7	10.2	2.0	11.2	2.0	12.6	0.0	0.0	1.1	3.6	2.0	7.2	1.4	9.4
IFNB1	3.5	8.8	3.8	14.6	4.7	15.1	4.5	11.6	4.6	13.7	0.0	0.0	3.6	13.7	1.0	3.5	1.5	7.6
IFNG	5.1	40.1	6.3	51.2	6.3	53.6	6.0	46.7	6.4	50.0	0.7	3.0	2.4	12.4	1.9	20.4	4.0	28.8
TNF	4.7	53.4	5.8	51.1	6.8	57.0	7.7	60.9	7.4	61.9	1.8	4.6	4.0	16.7	4.5	31.3	6.0	41.7

Note: The predicted activation state of upstream regulators is shown for the comparison of each HFD relative to chow (left half of table) and for the sunflower oil, pork fat, beef fat and milk fat diets relative to olive/rapeseed oil (right half of table). Regulators with a $-\log(p\text{-value}) > 2$ are considered to be significantly enriched. The *Z*-score indicates predicted activation (cutoff >2.0, marked in red) or inactivation (cutoff <−2, marked in green) of a regulator.

was more activated in the sunflower oil group), while the animal-fat diets further activated many of these cytokines relative to the olive/rapeseed oil group (for instance IL1A, IL1B, IL6, CSF2, and TNF)—an effect that was most pronounced for the milk fat diet.

3.6 | Specific inflammation markers and drivers of atherogenesis are differentially affected by the energy-dense high-fat diets

To gain more insight into possible inflammatory drivers of atherogenesis, we analyzed the plasma concentrations of the vascular adhesion molecules E-selectin, P-selectin, intercellular adhesion molecule 1 (ICAM) and vascular cell adhesion molecule 1 (VCAM. The energy-dense HFDs did not affect the levels of E-selectin and P-selectin in plasma (Figure 4A,B), while the plasma levels of VCAM and ICAM were significantly increased by all HFDs, with no differences between the diets (Figure 4C,D). Next we studied the potential involvement of the cytokine MIF, one of the proatherogenic cytokines identified in the transcriptomics analysis (Table 3), which is known to promote atherogenesis in Ldlr-deficient mice.[46] A correlation analysis revealed that there was a strong correlation between MIF concentrations and the total atherosclerotic lesion area (Figure 4E). We then compared plasma MIF levels between groups and found that in the chow group, plasma levels of the atherogenic cytokine MIF were low (Figure 4F). While the plant oil diets did not significantly increase plasma MIF relative to chow, the three animal-fat diets increased plasma MIF significantly—with an intermediate increase in the pork fat group and the most pronounced increases in the beef- and milk-fat groups.

4 | DISCUSSION

In this study, we show that changing the type of dietary fat in otherwise identical isocaloric diets has profound effects on atherosclerotic disease development in a translational diet-induced model of obesity-associated atherosclerosis. We compared five commonly consumed dietary fat sources: two plant-based fats (an olive/rapeseed oil mix and sunflower oil) and three animal fats (pork fat, beef fat, and milk fat).

Among the many known risk factors for CVD, diet is accepted to play an important role.[47] There is increasing evidence that different types of fat have divergent effects on health, but conflicting results have been the cause for much debate—both in the scientific community and the general public. Such contradictory results are most likely attributable to methodological issues that are inherent to both randomized controlled trials (the golden standard for establishing causality) and prospective cohort studies.[16,48] The

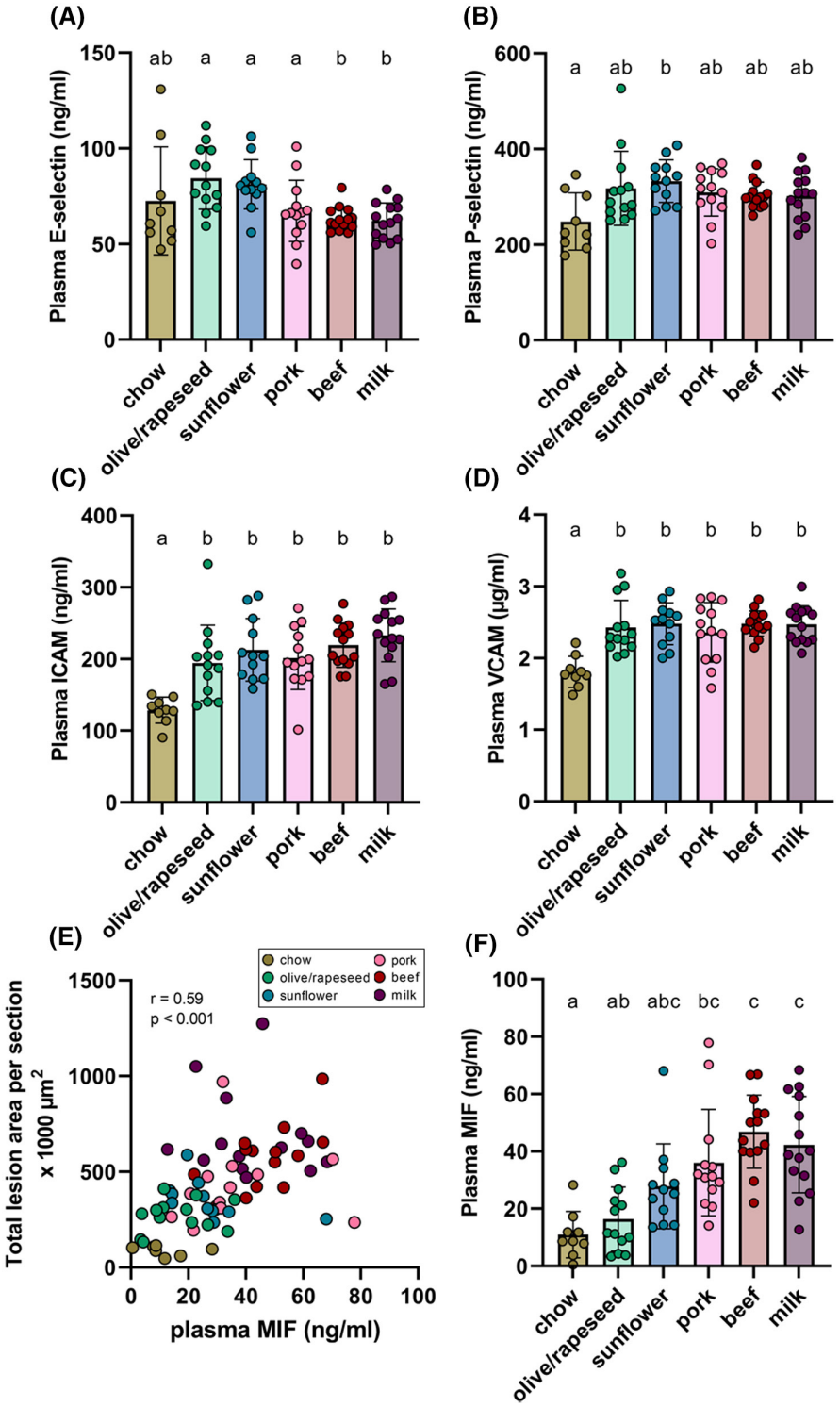

FIGURE 4 Effects of energy-dense high-fat diets (HFDs) on circulating (vascular) inflammation markers. Ldlr−/−.Leiden mice were fed energy-dense HFDs that were identical apart from their fat source for 28 weeks. (A) plasma E-selectin, (B) plasma P-selectin, (C) plasma ICAM and (D) plasma VCAM levels, (E) correlation between plasma macrophage migration inhibitory factor (MIF) and total atherosclerotic lesion area, (F) Plasma MIF levels, all measured in week 24 of the study. Data shown is mean ± SD. Bars with corresponding letters are statistically comparable ($p > .05$).

most crucial issues are the replacement macronutrient and the difficulty of isolating effects of specific macronutrients due to confounders (for instance due to changes in intake of fruit, vegetable or fiber intake). Overall, it appears that replacement of energy intake from saturated fats by mono-unsaturated or polyunsaturated fats reduces risk of CVD while replacement of saturated fats by carbohydrates has no effect on CVD risk if the replacement carbohydrates are refined whereas replacement of saturated fats with whole grains reduces disease risk.[15] While control of experimental conditions and avoidance of confounders is a challenge in human studies, animal studies do allow long-term studies of isolated dietary components while keeping the rest of the diet identical between experimental groups. Under these controlled experimental conditions with a duration roughly equivalent to 20 human-years, we were able to show that indeed atherosclerosis development was more pronounced with increasing SFA content in the diet and decreasing

MUFA and PUFA content. This was most clearly appreciable in the animal-fat diets, which are high in SFA and low in MUFA and PUFA relative to the vegetable oils. In addition, the trans fats that are present particularly in the beef and milk fat diets (and completely absent in the plant-derived oils) may have contributed to disease development although there is still some debate whether naturally occurring trans fatty acids (e.g., the ruminant trans fatty acid vaccenic acid, C18:1t) are as detrimental to cardiovascular health as trans fatty acids from industrial sources (i.e., those that are formed in the partial dehydrogenation of vegetable oils).[49,50]

One of the mechanisms by which dietary fats can influence CVD risk is through their effects on plasma cholesterol, an important driving force in atherosclerotic disease development. In the current study, we observed that while all energy-dense HFDs had significantly higher plasma triglycerides and total cholesterol levels than the low-fat chow diet, the SFA-rich animal fats more pronouncedly increased circulating lipids than the low-SFA plant-derived fats. This is in line with results from observational studies in humans that show increased plasma lipids with diets high in SFA.[51] In addition, the SFA-rich animal fats were higher in dietary cholesterol than the plant-derived fats, which can also have contributed to the observed differences in plasma cholesterol levels, together with the differences observed in fecal cholesterol excretion. Additionally, learance of cholesterol from the circulation may contribute to the observed differences in circulating cholesterol levels. Although there is no clearance of LDL particles via the LDL receptor in the Ldlr−/−.Leiden mouse due to the absence of a functional LDL receptor, there is a functional LRP1 receptor in the model, through which lipoprotein clearance can still take place. While there was a tendency toward inactivation of this LRP1 receptor by all HFDs relative to chow, only the milk fat diet reached the *z*-score cut-off of <−2, which may partly explain the increased plasma cholesterol levels observed in this group. The reduced clearance and accumulation of LDL particles is further supported by the significant increase ApoB concentrations with milk fat relative to chow and plant fats. Because each (V)LDL particle contains a single ApoB molecule, the absolute number of proatherogenic particles is highest with milk fat. In addition to this, the dietary fats had different effects on plasma triglyceride concentrations which were clearly higher with the animal fats. The relative change in triglycerides with animal fats was notably greater than would be expected from the relative increase in ApoB. Hence, this may suggest a reduced uptake of fatty acids from these particles into tissues that utilize or store fatty acids specifically in case of the animal fats. Since adipose tissue histology (e.g., hypertrophy and CLS analysis) did not reveal differences between the fats it is possible that the differential effects are at the level of tissue lipases. It is noteworthy to mention that oxLDL levels were comparable between the fats but that olive oil/rapeseed and milk fat showed significant elevations compared to chow. This may seem counterintuitive and may have different causes: the content of fatty acids with double bonds that are prone to oxidation was the highest in the olive oil/rapeseed group and we did not add antioxidants to prevent lipid peroxidation. In the case of the milk fat diet, LDL particles and fatty acids therein seem to be less efficiently utilized or cleared and possibly remain longer in the circulation (i.e., difference in LDL flux) where they become oxidized. Differences between dietary fats could be assessed by dynamical labeling studies to quantify LDL fluxes (e.g., residence time in circulation, oxidation, influx into vasculature), of which currently very little is known.

A second mechanism by which dietary fats can influence atherosclerotic disease development is through their effects on inflammation—a key driver of atherosclerosis that is independent of plasma cholesterol and other traditional risk factors.[52] Here, we show that different dietary fat sources differentially affect inflammation. Although we did not observe any differences between the energy-dense HFDs in adhesion molecule levels (i.e., circulating E-selectin, P-selectin, ICAM, and VCAM) we did find that the plasma levels of the pro-atherogenic chemotactic cytokine MIF[53] was significantly elevated only by the animal fat diets and not by the plant oil diets. This increased pro-inflammatory state in the animal fat diets relative to the plant oil diets was also reflected in the predicted activation state of upstream regulators in the liver. This analysis showed that although all of the energy-dense HFDs strongly activated pro-inflammatory signaling pathways, this activation was most pronounced in the animal fat diets (CCR2, MIF, IL1A and IL1B, IL6, CSF2, TNF, all showed significant activation in pork, beef and/or milk fat relative to the olive/rapeseed oil diet). Since fatty acids can affect inflammation via a number of routes, there are several mechanisms that may underlie these observations. Notably, many of these effects are local at the tissue level and not directly reflected in plasma. Firstly, SFAs can promote inflammation through TLR4-regulated mechanisms[54] providing a rationale for inducing inflammation by the animal-fat diets with higher SFA content. Secondly, polyunsaturated fatty acids are precursors for the synthesis of oxylipins, important inflammatory mediators that can have either pro-inflammatory or anti-inflammatory effects.[55,56] It has recently been shown for instance that dietary supplementation with an omega-3 fatty acid-rich krill oil affects the oxylipin balance in adipose tissue and liver. Omega-3 fatty acid-derived oxylipins (anti-inflammatory) were elevated and arachidonic acid (omega-6) oxylipins (pro-inflammatory) were reduced[34] which overall was associated with reduced inflammation in these tissues. It is conceivable that similar effects

may occur in vascular tissue based on previous reports demonstrating an important role for oxylipins in vascular inflammation.[57] Cholesterol-independent effects of such mediators on atherosclerosis have also been reported.[58] Differences in the PUFA content and composition of the experimental diets in the current study may thus have differentially affected the balance of pro- and anti-inflammatory oxylipins within the vasculature, thereby contributing to the observed differences in atherosclerotic disease development. Lastly, fatty acids can also serve as the ligand for ligand-activated transcription factors of the peroxisome proliferator-activated receptor (PPAR) family. In addition to their important role in the regulation of metabolic processes, these transcription factors are also known to affect inflammation through transrepression of the activity of many inflammatory transcriptional regulators such as NF-κB, STATs and NFAT.[59] As MUFA and PUFA are more potent PPAR ligands than SFA, the inflammation-modulating properties of PPARs are differentially modulated by diets that differ in fatty acid composition. Furthermore, it is possible that independent of the fatty acid composition of the tested dietary fats, other components (e.g., plant sterols or phytochemicals) especially in the plant fats may also have anti-inflammatory and atheroprotective properties.[60,61] Conversely, the cholesterol present in the dietary fats (particularly in the beef and milk fat) may have pro-inflammatory and pro-atherogenic effects.[62]

Altogether, our study shows that different dietary fats sources have differential effects on atherosclerotic disease development. Importantly, we demonstrate this in a translational model for obesity-associated atherosclerotic disease in a highly controlled experimental setting, in which the type of dietary fat was the only factor that differed between the experimental diets used. We show that atherosclerosis development is more pronounced with increasing dietary SFA and cholesterol content and decreasing dietary MUFA and PUFA content and is associated with increases in proatherogenic plasma lipids/lipoproteins as well as proatherogenic mediators of inflammation such as MIF.

AUTHOR CONTRIBUTIONS

Harald Carlsen, Bjørg Egelandsdal and Robert Kleemann conceived and designed the research. Sérgio D. C. Rocha, Harald Carlsen, Bjørg Egelandsdal and Silje Harvei, performed the in vivo experiment, organized samples, and analyzed the adipose tissue. Elsbet J. Pieterman and Robert Kleemann coordinated and acquired the biochemical data (plasma lipids, NGS) and atherosclerosis. Martine C. Morrison, Elsbet J. Pieterman, Harald Carlsen, Bjørg Egelandsdal, and Robert Kleemann analyzed and interpreted the data. All authors were involved in drafting and revising the manuscript.

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DATA AVAILABILITY STATEMENT

The gene expression data from the hepatic transcriptomics analysis has been made available online in the Gene Expression Omnibus (GEO) repository (https://www.ncbi.nlm.nih.gov/geo/) under accession number GSE232133. All other data that support the findings of this study are available in the methods, results and/or supplementary material of this article.

DISCLOSURES

The authors declare no conflicts of interest.

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