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Abstract: Background and aim:

Oxidative modification of lipoproteins is a crucial step in atherosclerosis development. Isotopic-reinforced polyunsaturated fatty acids (D-PUFAs) are more resistant to reactive oxygen species-initiated chain reaction of lipid peroxidation than regular hydrogenated (H-)PUFAs. We aimed to investigate the effect of D-PUFA treatment on lipid peroxidation, hypercholesterolemia and atherosclerosis development.

Methods:

Transgenic APOE\*3-Leiden.CETP mice, a well-established model for human-like lipoprotein metabolism, were pre-treated with D-PUFAs or control H-PUFAs-containing diet (1.2%, w/w) for 4 weeks. Thereafter, mice were fed a Western-type diet (containing 0.15% cholesterol, w/w) for another 12 weeks, while continuing the D-/H-PUFA treatment.

Results:

D-PUFA treatment markedly decreased hepatic and plasma F2-isoprostanes (approx. -80%) and prostaglandin F2 $\alpha$  (approx. -40%) as compared to H-PUFA treatment. Moreover, D-PUFAs reduced body weight gain during the study (-54%) by decreasing body fat mass gain (-87%) without altering lean mass. D-PUFAs consistently reduced plasma total cholesterol levels (approx. -25%), as reflected in reduced plasma non-HDL-cholesterol (-28%). Additional analyses of hepatic cholesterol metabolism indicated that D-PUFAs reduced the hepatic cholesterol content (-21%). Sterol markers of intestinal cholesterol absorption and cholesterol breakdown were decreased. Markers of cholesterol synthesis were increased. Finally, D-

PUFAs reduced atherosclerotic lesion area formation throughout the aortic root of the heart (-26%).

Conclusions:

D-PUFAs reduce body weight gain, improve cholesterol handling and reduce atherosclerosis development by reducing lipid peroxidation and plasma cholesterol levels. D-PUFAs therefore represent a promising new strategy to broadly reduce rates of lipid peroxidation, and combat hypercholesterolemia and cardiovascular diseases.

## Highlights

### Highlights

- D-PUFAs decrease lipid peroxidation products in hyperlipidemic mice.
- D-PUFAs reduce body weight and body fat mass gain.
- D-PUFAs lower plasma cholesterol levels and improve cholesterol handling.
- D-PUFAs reduce atherosclerotic lesion formation.

**Deuterium-reinforced polyunsaturated fatty acids protect against atherosclerosis by lowering lipid peroxidation and hypercholesterolemia**

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**Methods:** Transgenic *APOE\*3-Leiden.CETP* mice, a well-established model for human-like lipoprotein metabolism, were pre-treated with D-PUFAs or control H-PUFAs-containing diet (1.2%, w/w) for 4 weeks. Thereafter, mice were fed a Western-type diet (containing 0.15% cholesterol, w/w) for another 12 weeks, while continuing the D-/H-PUFA treatment.

**Results:** D-PUFA treatment markedly decreased hepatic and plasma  $F_2$ -isoprostanes (approx. -80%) and prostaglandin  $F_2\alpha$  (approx. -40%) as compared to H-PUFA treatment. Moreover, D-PUFAs reduced body weight gain during the study (-54%) by decreasing body fat mass gain (-87%) without altering lean mass. D-PUFAs consistently reduced plasma total cholesterol levels (approx. -25%), as reflected in reduced plasma non-HDL-cholesterol (-28%). Additional analyses of hepatic cholesterol metabolism indicated that D-PUFAs reduced the hepatic cholesterol content (-21%). Sterol markers of intestinal cholesterol absorption and cholesterol breakdown were decreased. Markers of cholesterol synthesis were increased. Finally, D-PUFAs reduced atherosclerotic lesion area formation throughout the aortic root of the heart (-26%).

**Conclusions:** D-PUFAs reduce body weight gain, improve cholesterol handling and reduce atherosclerosis development by reducing lipid peroxidation and plasma cholesterol levels. D-PUFAs therefore represent a promising new strategy to broadly reduce rates of lipid peroxidation, and combat hypercholesterolemia and cardiovascular diseases.

## 1. Introduction

Atherosclerotic vascular disease, comprising heart attacks, stroke, aortic aneurysms, and peripheral vascular disease, is the most frequent cause of death in the Western world [1,2]. The impact of the atherosclerosis pandemic is predicted to increase worldwide over the next few decades, despite recent progress in lipid-lowering therapy [2,3].

Increased retention of low-density lipoprotein (LDL) in the vessel wall and subsequent oxidative modification is a crucial step in the pathogenesis of atherosclerosis [4]. Polyunsaturated fatty acids (PUFAs) can get oxidized through either enzymatic or non-enzymatic pathways. Non-enzymatic damage can be initiated by both 2-electron- and 1-electron-oxidants, through an addition to a double bond, or, more typically (mostly for 1-electron-oxidants) by H-atom abstraction off a *bis*-allylic methylene group. Once the radical is formed, the ensuing chain reaction of lipid peroxidation (LPO) multiplies the destruction. A smorgasbord of the downstream products of LPO include toxic carbonyls which further exacerbate the atherosclerosis-related damage, through the modification of lipids [5] and apoB within LDL [6] (Figure 1). Aldehyde-modified LDL-apoB is scavenged by macrophages in an uncontrolled manner leading to foam cell formation and the initiation of the atherosclerotic lesion [6]. Reactive carbonyls as well as oxysterols, cholesterol oxidation products, contribute to atherosclerosis mainly through their ability to induce inflammation, oxidative stress and apoptosis. [7,8]. Isoprostanes are another LPO-derivative from PUFA oxidation (Fig. 1), although some (*e.g.* 8-iso-prostaglandin-F<sub>2</sub>) can also be produced enzymatically. These bioactive molecules can promote atherosclerosis development among others via inducing inflammation and enhancing endothelial/immune cell interaction [9].

Yet, oral antioxidants have not provided the obvious solution to this LPO problem. The most likely reason for this is that it has proven impractical to supplement an organism with sufficient antioxidants in order to block ongoing LPO chain reactions [10,11], specifically when endogenous protective antioxidant mechanisms are disrupted due to other underlying clinical conditions. In addition, some antioxidants may induce adverse effects as for instance, in the presence of an excess of LDL, vitamin E ( $\alpha$ -tocopherol) may act as a pro-oxidant [12,13].

PUFAs are essential nutrients as they are not synthesized in mammalian tissues and have to be supplied through the diet. Following ingestion, PUFAs are quickly incorporated into lipid structures throughout the body. Deuterium is a stable hydrogen isotope that has natural abundance (150 ppm in ocean water) and is accordingly recognized by living systems as a normal, natural sub-type of hydrogen. Deuterium incorporated into PUFAs at bis-allylic positions (D-PUFAs) gives rise to a well-known “kinetic isotope effect” [14] as a result of which, reactions involving cleavage of a C-H bond are slowed down in the C-D bond. As the abstraction step by reactive oxygen species (ROS) is repeated throughout the chain of LPO events, the protective effect of D-PUFAs is multiplied, thus resulting in a larger total beneficial effect when compared to exposure with normal (H-)PUFAs. As a result we have been able to show that D-PUFAs, that are specifically deuterated at the bis-allylic positions,

are resistant to LPO [15-18], and can mitigate several pathologies, including important aspects of cellular damage in Friedreich's ataxia [19] and Parkinson's disease [20,21].

As oxidative stress is a crucial step in atherosclerosis development and there is likely interplay between oxidative stress and lipid metabolism, we aimed to investigate the effect of D-PUFA treatment on LPO, hypercholesterolemia and atherosclerosis development in *APOE\*3-Leiden.CETP* mice. This hyperlipidemic model is a well-established model for human-like lipoprotein metabolism. Unlike hyperlipidemic apoE- and LDLR-deficient mice, they have an intact, albeit attenuated, apoE-LDLR clearance pathway for cholesterol-enriched lipoprotein remnants [22,23]. As a result *APOE\*3-Leiden.CETP* mice respond well to lipid-lowering and anti-atherogenic effects of e.g. statins [24], PCSK9 inhibition [25] and niacin [26]. Our results show that treatment with D-PUFAs markedly reduces the production of LPO products and plasma cholesterol levels thereby lowering the development of atherosclerosis.

## 2. Materials and Methods

### *Mice, diets and general animal procedures*

*APOE\*3-Leiden* mice were crossbred with mice expressing human cholesteryl ester transfer protein (CETP) under control of its natural flanking regions to generate heterozygous *APOE\*3-Leiden.CETP* mice [22]. Female *APOE\*3-Leiden.CETP* mice of 10–12 weeks of age were housed under standard conditions (3–4 mice per cage) with a 12-h light/dark cycle and free access to food and water. At  $t=-4$  weeks, mice were randomized based on 4-hour fasted plasma lipids, age, body weight and body composition and divided into 2 groups.

Subsequently, mice were fed an AIN-93M-based ‘preloading’ diet (Research Diets, USA) containing either control PUFAs (H-PUFA group) or the isotope-reinforced PUFAs (D-PUFA group) for incorporation of the PUFAs in the body. No cholesterol was added yet to these preloading diets. The composition of all diets is presented in Supplemental Table 1. Specifically, these diets comprised 12% fat, which contained per fat fraction 65% saturated fatty acids (coconut oil 101, hydrogenated), 25% monounsaturated fatty acids (ethyl oleate), and 10% PUFAs (ethyl linoleate and ethyl linolenate in an 1:1 weight ratio; control H-PUFA group). In the D-PUFA group the H-PUFAs were replaced by isotope-reinforced D-PUFAs (ethyl 11,11-D<sub>2</sub>-linoleate and ethyl-11,11,14,14-D<sub>4</sub>-linolenate in a 1:1 weight ratio). After 4 weeks of feeding on this preloading diet, at  $t=0$ , the diets of the mice were switched to the same diets as above, but now containing 0.15% cholesterol (‘Western-type diet’, WTD) for 12 weeks to induce development of atherosclerotic lesions. Food intake was monitored per cage twice per week. Body weight as well as total body fat and lean mass by EchoMRI-100 (EchoMRI, Houston, Texas), were determined every 4 weeks during the study. Animal experiments were approved by the Ethics Committee on Animal Care and Experimentation of the Leiden University Medical Center.

The full Materials and Methods section is available in the Supplemental Materials and Methods.



### 3. Results

#### 3.1. D-PUFAs efficiently incorporate into tissues

Female *APOE\*3-Leiden.CETP* mice were pre-treated with preloading diets containing either isotope-reinforced D-PUFAs or control H-PUFAs (composition of the diets is presented in Supplemental Table 1). After 4 weeks at t=0, cholesterol was added to these diets to induce hyperlipidemia and atherosclerosis development. At the end of the study, 12 weeks after Western-type diet (WTD)-feeding efficient D-PUFA incorporation was confirmed by measuring deuterium content on sections of the tails of mice. The difference between the D-PUFA ( $6,982 \pm 217\%$ ) and the H-PUFA ( $-57 \pm 5\%$ ;  $p < 0.001$ ) group corresponds to approx. 30% D-PUFA incorporation (*i.e.* D-PUFA fraction of total PUFA) based on previous studies [20,27]. This level of D-PUFA substitution is biologically relevant as approx. 10-20% is sufficient to terminate LPO [16].

#### 3.2. D-PUFAs reduce lipid peroxidation products

As oxidative stress is a main contributor to the development of atherosclerosis and D-PUFAs have been previously shown to reduce LPO products in other models [15-20], we investigated whether D-PUFA treatment also reduces LPO in hyperlipidemic *APOE\*3-Leiden.CETP* mice. Indeed, D-PUFA treatment markedly decreased F<sub>2</sub>-isoprostanes (F<sub>2</sub>-IsoPs; -72%) and prostaglandin F<sub>2</sub> $\alpha$  (PGF<sub>2</sub> $\alpha$ ; -44%) levels in the liver (Fig. 2A). More importantly with respect to atherosclerosis development, D-PUFAs also markedly reduced plasma levels of F<sub>2</sub>-isoprostanes (-87%) and PGF<sub>2</sub> $\alpha$  (-40%) (Fig. 2B). D-PUFA treatment did not affect the liver toxicity markers ALAT and ASAT in plasma (not shown). These findings indicate that D-PUFAs potently reduce tissue and circulating LPO products in hyperlipidemic *APOE\*3-Leiden.CETP* mice, without adverse effects on the liver.

#### 3.3. D-PUFAs reduce body fat mass, while enhancing food intake

To study the effect of D-PUFAs on the metabolic phenotype of mice, body weight and body composition were monitored during the study. D-PUFAs reduced body weight (up to -9%; Fig. 3A) and body weight gain (up to -54%; Fig. 3B) during the study. D-PUFAs did not consistently affect lean mass gain (Fig. 3C), but effectively prevented body fat mass gain (up to -87%; Fig. 3D). Accordingly, the weight of gonadal white adipose tissue (gWAT) was reduced in the D-PUFA group as compared to the control H-PUFA group (-60%; Fig. 3E). These beneficial effects on body fat mass were not the result of decreased food intake as daily food intake even gradually increased in the D-PUFA group after 5.5 weeks of cholesterol-feeding (up to +33% after 12 weeks; Fig. 3F).

#### 3.4. D-PUFAs lower plasma cholesterol levels and alter hepatic cholesterol homeostasis

Subsequently, we assessed the effect of D-PUFA treatment on lipid metabolism, in particular cholesterol metabolism, the main contributor to atherosclerosis development. D-PUFAs did not modulate fasting plasma triglyceride (TG) levels up to t=4 weeks, but did increase plasma TG levels (up to +36%; Fig 4A) in the last phase of the study when food intake was also increased. Under non-cholesterol-feeding conditions, D-PUFAs minimally increased fasting plasma total cholesterol (TC) levels (+14%; Fig. 4B). However, under cholesterol-feeding

hypercholesterolemic conditions, D-PUFAs consistently reduced plasma TC levels (ranging from -20% to -37%). Combined, D-PUFA treatment resulted in clearly reduced plasma total cholesterol exposure during the complete study (-23%; Fig. 4C). The reduced plasma TC level (-4.6 mM at the endpoint) was confined to both reduced plasma non-HDL-C (-4.0 mM; -28%; **calculated as TC minus HDL-C**) and reduced plasma HDL-C (-0.7 mM; -48%) levels (Fig. 4D).

As D-PUFAs reduced plasma cholesterol levels we studied the effect of D-PUFA treatment on cholesterol metabolism in more detail by quantifying hepatic TC content as well as hepatic sterol markers of intestinal cholesterol absorption (*i.e.* cholestanol, campesterol, sitosterol), cholesterol synthesis (*i.e.* desmosterol, lathosterol, lanosterol, dihydro-lanosterol) and cholesterol breakdown (*i.e.* 24OH-cholesterol, 7 $\alpha$ OH-cholesterol, 27OH-cholesterol). D-PUFAs reduced the hepatic TC content (-21%; Fig. 4E). Interestingly, this was accompanied with reduced intestinal cholesterol absorption markers (*i.e.* cholestanol, campesterol) in the liver (Fig. 4F). Moreover, D-PUFAs enhanced hepatic markers of cholesterol synthesis (*i.e.* desmosterol, lathosterol, dihydro-lanosterol; Fig. 4G), while also reducing most markers of cholesterol breakdown (*i.e.* 24OH-cholesterol and 7 $\alpha$ OH-cholesterol were reduced, 27OH-cholesterol was increased; Fig. 4H). These data together indicate that D-PUFAs influence cholesterol metabolism, eventually resulting in a reduced hepatic cholesterol content and reduced plasma non-HDL-C levels.

### 3.5. D-PUFAs reduce atherosclerotic lesion development

Finally, we investigated whether the D-PUFA-mediated lowering of LPO and hypercholesterolemia resulted in reduced atherosclerosis development. Indeed, treatment with D-PUFAs reduced atherosclerotic lesion area throughout the aortic root of the heart (ranging from -23% to -30%; Fig. 5A-B), resulting in 26% lower mean atherosclerotic lesion area in the D-PUFA-treated mice as compared to H-PUFA-treated mice ( $79 \pm 9$  vs.  $107 \pm 12 \times 10^3 \mu\text{m}^2/\text{cross section}$ , respectively; Fig. 5C). Taken together, these findings demonstrate that D-PUFAs lower LPO as well as hypercholesterolemia, ultimately resulting in reduced atherosclerosis development in *APOE\*3-Leiden.CETP* mice.

#### 4. Discussion

Isotope-reinforced D-PUFAs are resistant to non-enzymatic LPO and effectively reduce oxidative stress in various experimental models [15-20], but their effect on lipid metabolism and atherosclerosis development had not been investigated before. In the present study, we show that dosing with ethyl esters of 11,11-D2-linoleic acid and 11,11,14,14-D4-linolenic acid markedly reduced LPO products under hypercholesterolemic conditions in *APOE\*3-Leiden.CETP* mice. D-PUFAs also reduced body weight gain by reducing body fat mass, under conditions of increased food intake. Moreover, D-PUFAs reduced plasma TC, confined to both non-HDL-C and HDL-C. As a result of these anti-atherogenic characteristics, D-PUFAs reduced atherosclerosis development. Our study sheds light on the therapeutic and atheroprotective opportunity afforded by oral dosing with D-PUFAs.

D-PUFAs were efficiently incorporated in the body. More importantly, this level of incorporation was sufficient to markedly reduce hepatic and circulating LPO products, as measured by F<sub>2</sub>-IsoPs, under hypercholesterolemic conditions, in line with previous studies in yeast [16] and normolipidemic mice [20]. It is important to note that F<sub>2</sub>-IsoPs are products resulting from the non-enzymatic oxidation of arachidonic acid. Arachidonic acid can also undergo enzymatic oxidation to yield PGF<sub>2</sub> $\alpha$  as well as other products by COX-1 in platelets and by COX-2 in monocytes [28]. Comparing levels of F<sub>2</sub>-IsoPs (formed primarily by non-enzymatic LPO) with those of PGF<sub>2</sub> $\alpha$  (formed primarily by enzymatic LPO, *i.e.* by COX enzymes) reveals that while non-enzymatic LPO is substantially down-regulated in the D-PUFA group, the enzymatic oxidation is much less affected (Fig. 2). That D-PUFAs also reduce enzymatic oxidation to some extent can be explained by the fact that 11,11-D2-linoleic acid is efficiently converted to 13,13-D2-arachidonic acid. The presence of the deuterium at carbon 13 on arachidonic acid will influence both the non-enzymatic and enzymatic oxidation as this is the site of action of the COX enzymes. Non-enzymatic oxidation is also influenced by the presence of other D-PUFAs in lipid membranes slowing down the free radical chain reaction and resulting in a greatly decreased level of F<sub>2</sub>-IsoPs. D-PUFAs thus have anti-atherogenic potential in hypercholesterolemic mice by reducing systemic, and likely also local, non-enzymatic oxidative stress.

Besides reducing oxidative stress, we observed that treatment with D-PUFAs reduced body weight gain as a result of reduced body fat gain. This was not the result of reduced food intake, in fact, food intake gradually increased over the course of the last 7 weeks. As a result, the D-PUFA cohort were exposed to more atherogenic dietary cholesterol, making the findings of reduced lesions and blood cholesterol even more profound. It is likely also not the result of reduced growth as lean mass was not different between the groups. The reduced body fat mass is in line with previous studies showing that reducing oxidative stress reduces obesity [29-30], but studies on potential underlying mechanism(s) are scarce. Therefore, the main mechanism(s) underlying the D-PUFA-mediated reduced fat mass gain remains to be identified, but may include reduced adipocyte differentiation as was observed in COX-2-deficient mice [31]. Overall, D-PUFAs thus beneficially influence the body composition despite increased food intake.

During cholesterol-feeding, D-PUFAs consistently reduced fasted plasma TC levels. This reduction was mainly attributed to reduced non-HDL-C levels and was also

1 accompanied by a reduced hepatic cholesterol content at the end of the study. In-depth  
2 analyses of hepatic cholesterol metabolism showed that D-PUFAs reduce markers of  
3 cholesterol absorption, indicative of reduced intestinal cholesterol absorption. This reduced  
4 intestinal cholesterol absorption is likely key to the D-PUFA-mediated beneficial effects on  
5 cholesterol metabolism. First, the cholesterol-lowering effects of D-PUFAs are dependent on  
6 dietary cholesterol as plasma TC levels are decreased only during cholesterol-feeding and not  
7 under non-cholesterol-feeding conditions. Second, it is well-known that inhibition of  
8 cholesterol absorption via genetic (*i.e.* Niemann-Pick C1 like 1-deficiency) or  
9 pharmacological (*i.e.* ezetimibe) modulation reduces the hepatic cholesterol content and  
10 plasma non-HDL-C levels in hyperlipidemic mice [32,33]. Our results indicate that this is a  
11 cholesterol-specific effect and not the result of a general reduction in chylomicron production  
12 and secretion, as plasma TG levels are unaffected or even increased. Reports on modulation  
13 of oxidative stress or inflammation in relation to intestinal cholesterol absorption are scarce.  
14 Interestingly, Stöger *et al.* [34] recently reported an anti-inflammatory mouse model with  
15 reduced intestinal cholesterol absorption and plasma TC levels, but the underlying  
16 mechanism(s) remain obscure. As treatment with D-PUFAs affect many mediators and  
17 possibly thus many processes, the exact mechanism(s) underlying the reduced intestinal  
18 cholesterol absorption upon D-PUFA treatment may be complex. Potential mechanism(s)  
19 may include modulation of the gut microbiome and/or bile acid metabolism in the gut  
20 [35,36], and modulation of (local) immune cells [37,38]. For example, dendritic cells, which  
21 are present in the inner lining of the intestines, may play a key role. Antioxidative strategies  
22 protect dendritic cells against degeneration [39], and increasing the lifespan and  
23 immunogenicity of dendritic cells reduces plasma cholesterol levels (via an unexplored  
24 mechanism) [37]. Together, one could speculate that D-PUFAs improve the lifespan and  
25 immunogenicity of the dendritic cells in the intestines, thereby reducing dietary cholesterol  
26 absorption and reducing plasma cholesterol levels. However, more detailed studies are  
27 required.

28 We also observed that D-PUFAs increase hepatic cholesterol synthesis and reduce  
29 hepatic cholesterol breakdown. While D-PUFAs reduced the cholesterol breakdown markers  
30 24OH-cholesterol and 7 $\alpha$ OH-cholesterol, 27OH-cholesterol levels were increased. The latter  
31 is possibly the result of CYP7A1 down-regulation, and may thus also be indicative of  
32 reduced cholesterol breakdown. These effects on hepatic cholesterol synthesis and cholesterol  
33 breakdown are likely secondary to the reduced intestinal cholesterol absorption in an attempt  
34 to maintain homeostasis. Hepatic cholesterol synthesis is well-known to increase upon  
35 reduced intestinal cholesterol absorption [32,33]. Presumably, D-PUFA treatment also  
36 decreases plasma non-HDL-C mainly as a consequence of the reduced intestinal cholesterol  
37 absorption and subsequent reduced hepatic cholesterol content. It is known that reducing the  
38 hepatic cholesterol content, for example by ezetimibe or statins, enhances the hepatic LDL  
39 receptor expression, consequently lowering plasma non-HDL-C levels [33]. However, also  
40 potential local antioxidative effects of D-PUFAs may have influenced these hepatic pathways  
41 of cholesterol synthesis and cholesterol breakdown directly. As outlined above for the  
42 intestinal cholesterol absorption, D-PUFAs affect many mediators and possibly thus many  
43 processes, and the exact underlying mechanism(s) may be complex. Taken together, our  
44 findings indicate that D-PUFAs lower the hepatic cholesterol content by reducing the

1 intestinal cholesterol absorption and possibly by additional (local) antioxidative effects,  
2 thereby ultimately reducing plasma non-HDL-C levels.

3 Finally, D-PUFA treatment reduced atherosclerotic lesion formation, which is in line  
4 with the reduced **systemic** oxidative and inflammatory status and reduced plasma non-HDL-  
5 C levels. Previous experimental studies also show that antioxidant strategies such as with  
6 vitamin E [40,41] and probucol [42] in most cases reduce atherosclerosis development in  
7 rodent models. However, numerous large clinical trials with antioxidants (typically vitamin E  
8 or beta carotene) in atherosclerosis have been consistently negative [43]. This does not  
9 necessarily challenge the importance of oxidative modification of LDL in the pathogenesis of  
10 atherosclerosis as well as the clinical potential of D-PUFA treatment. Rather, the choice or  
11 dosage of antioxidants could have been suboptimal, particular in view of a still unresolved  
12 issue of the relative importance of enzymatic versus non-enzymatic, as well as two-electron  
13 versus one-electron processes in LDL oxidation [13,44]. Indeed tocopherol, an accepted  
14 lipophilic chain-terminating antioxidant, can actually serve as a pro-oxidant in an LDL-rich  
15 environment, thus exacerbating the generation of LPO [12,13]. We propose that specific  
16 inhibition of non-enzymatic LPO, *e.g.* by D-PUFAs, may prove to be beneficial when  
17 targeting atherosclerosis and this process is likely superior to general **antioxidant-based**  
18 inhibition or **employing nonsteroidal anti-inflammatory drugs (NSAIDs)**. It is important to  
19 realize that D-PUFAs are not antioxidants: they are not quenching the ROS and do not  
20 disturb the redox status or antioxidant-ROS ratio, and so are unlikely to disturb normal ROS-  
21 mediated signalling pathways. Our results thus show that D-PUFAs reduce experimental  
22 atherosclerosis development by reducing both LPO and hypercholesterolemia. D-PUFAs may  
23 hence represent a promising alternative approach to reduce oxidative stress, including  
24 reducing the risk factors for, hypercholesterolemia and atherosclerosis in humans.

25 The limitations of the current study include a need to better understand how D-PUFAs  
26 reduce intestinal cholesterol absorption. In addition, the effects of D-PUFAs on body fat mass  
27 gain, while increasing food intake, is not well understood. Further studies are warranted to  
28 resolve the potential use of D-PUFAs for obesity-related therapeutic indications. A strength  
29 of our study is that we made use of isotopic-reinforced PUFAs instead of conventional  
30 antioxidant therapies which so far have failed therapeutically. Furthermore, we made use of  
31 hyperlipidemic *APOE\*3-Leiden.CETP* mice which have a lipoprotein metabolism that  
32 closely resembles that of humans.

33 In conclusion, our data demonstrate that D-PUFAs limit body weight gain, improve  
34 cholesterol handling and reduce the development of atherosclerotic lesions by reducing  
35 **systemic, and likely** local, LPO and by reducing plasma non-HDL-C levels. The reduced  
36 plasma non-HDL-C levels probably mainly result from reduced intestinal cholesterol  
37 absorption, though additional mechanisms cannot be excluded. Future studies should  
38 elucidate whether D-PUFA treatment also reduces LPO, hypercholesterolemia and  
39 cardiovascular disease risk in humans.

## 5. Conflict of interest

Financial support and D-PUFA reagents were provided by Retrotope, Inc. Lex H.T. van der Ploeg and Mikhail S. Shchepinov receive compensation from and hold stock in Retrotope, Inc. The other authors have no conflicts to declare.

## 6. Author contributions

J.F.P.B., P.C.N.R. and M.S.S. designed the experiments with the help from I.M.M. and L.H.T.v.d.P. Experiments were performed and data analysed by J.F.P.B., I.M.M., G.L.M., E.P., G.H. and D.L. Data were interpreted by J.F.P.B., D.L. and P.C.N.R. M.S.S., C.M. and L.H.T.v.d.P provided intellectual contributions throughout the project. J.F.P.B. and M.S.S. wrote the manuscript. All authors discussed the results and commented on the manuscript.

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## 8. References

1. E. Brawnwald, Cardiovascular medicine at the turn of the millennium: triumphs, concerns, and opportunities, *N. Engl. J. Med.* 337 (1997) 1360-1369.
2. R. Chou et al., Statins for prevention of cardiovascular disease in adults: evidence report and systematic review for the US preventive services task force, *JAMA* 316 (2016) 2008-2024.
3. S. Bansilal et al., Global burden of CVD: focus on secondary prevention of cardiovascular disease, *Int. J. Cardiol.* 201 (2015) S1-S7.
4. K.J. Williams and I. Tabas, The response-to-retention hypothesis of atherogenesis reinforced, *Curr. Opin. Lipidol.* 9 (1998) 471-474.
5. V. Serbulea et al., The effect of oxidized phospholipids on phenotypic polarization and function of macrophages, *Free Radic. Biol. Med.* (2017), *in press*.
6. H. Esterbauer et al., Lipid peroxidation and its role in atherosclerosis, *Br. Med. Bull.* 49 (1993) 566-576.
7. S. Gargiulo et al., Oxysterols and 4-hydroxy-2-nonenal contribute to atherosclerotic plaque destabilization, *Free Radic. Biol. Med.* (2017), *in press*.
8. A. Negre-Salvayre et al., Proatherogenic effects of 4-hydroxynonenal, *Free Radic. Biol. Med.* (2017), *in press*.
9. J. Bauer, et al., Pathophysiology of isoprostanes in the cardiovascular system: implications of isoprostane-mediated thromboxane A2 receptor activation, *Br. J. Pharmacol.* 171 (2014) 3115-3131.
10. L. Packer, Antioxidant action of carotenoids in vitro and in vivo and protection against oxidation of human low-density lipoproteins, *Annals New York Acad. Sci.* 691 (1993) 48-60.
11. A.K. Jain et al., Role of antioxidants for the treatment of cardiovascular diseases: challenges and opportunities, *Curr. Pharm. Des.* 21 (2015) 4441-4455.
12. V.W. Bowry et al., Vitamin E in human low-density lipoprotein. When and how this antioxidant becomes a pro-oxidant, *Biochem. J.* 288 (1992) 341-344.
13. M.E. Lönn et al., Actions of "antioxidants" in the protection against atherosclerosis, *Free Radic. Biol. Med.* 53 (2012) 863-884.
14. F.H. Westheimer, The magnitude of the primary kinetic isotope effect for compounds of hydrogen and deuterium, *Chem. Rev.* 61 (1961) 265-273.
15. S. Hill et al., Isotope-reinforced polyunsaturated fatty acids protect yeast cells from oxidative stress, *Free Rad. Biol. Med.* 50 (2011) 130-138.
16. S. Hill et al., Small amounts of isotope-reinforced polyunsaturated fatty acids suppress lipid autoxidation, *Free Rad. Biol. Med.* 53 (2012) 893-906.
17. A.Y. Andreyev et al., Isotope-reinforced polyunsaturated fatty acids protect mitochondria from oxidative stress, *Free Rad. Biol. Med.* 82 (2015) 63-72.
18. C.R. Lamberson et al., Unusual kinetic isotope effects of deuterium reinforced polyunsaturated fatty acids in tocopherol-mediated free radical chain oxidations, *J. Amer. Chem. Soc.* 136 (2014) 838-841.
19. M.G. Cotticelli et al., Insights into the role of oxidative stress in the pathology of Friedreich ataxia using peroxidation resistant polyunsaturated fatty acids, *Redox Biol.* 1 (2013) 398-404.
20. M.S. Shchepinov et al., Isotopic reinforcement of essential polyunsaturated fatty acids diminishes nigrostriatal degeneration in a mouse model of Parkinson's disease, *Toxicol. Lett.* 207 (2011) 97-103.
21. P.R. Angelova et al., Lipid peroxidation is essential for alpha-synuclein-induced cell death, *J. Neurochem.* 133 (2015) 582-589.

22. M. Westerterp et al., Cholesteryl ester transfer protein decreases high-density lipoprotein and severely aggravates atherosclerosis in APOE\*3-Leiden mice, *Atheroscler. Thromb. Vasc. Biol.* 26 (2006) 2552-2559.
23. J.F. Berbée et al., Brown fat activation reduces hypercholesterolaemia and protects from atherosclerosis development, *Nat. Commun.* 6 (2015) 6356.
24. W. de Haan et al., Torcetrapib does not reduce atherosclerosis beyond atorvastatin and induces more proinflammatory lesions than atorvastatin, *Circulation* 117 (2008) 2515-2522.
25. S. Kühnast et al., Alirocumab inhibits atherosclerosis, improves the plaque morphology, and enhances the effects of a statin, *J. Lipid Res.* 55 (2014) 2103-2112.
26. S. Kühnast et al., Niacin reduces atherosclerosis development in APOE\*3 Leiden.CETP mice mainly by reducing nonHDL-cholesterol, *PLoS One* 8 (2013) e66467.
27. M.S. Shchepinov et al., Deuterium protection of polyunsaturated fatty acids against lipid peroxidation: a novel approach to mitigating mitochondrial neurological diseases, in: R.S. Watson, F. De Meester (Eds.), *Omega 3 Fatty Acids in Brain and Neurological Health*, Elsevier, Academic Press, 2014, pp. 373-383.
28. I. Tabas, Lipids and atherosclerosis, in: D.E. Vance and J.E. Vance (Eds.), *Biochemistry of Lipids, Lipoproteins and Membranes* (5<sup>th</sup> Edn.), Elsevier B.V. 2008, pp. 579-605.
29. B. Feng et al., Silymarin alleviates hepatic oxidative stress and protects against metabolic disorders in high-fat diet-fed mice, *Free Radic. Res.* 50 (2016) 314-327.
30. I. Sinha-Hikim et al., A novel cystine based antioxidant attenuates oxidative stress and hepatic steatosis in diet-induced obese mice, *Exp. Mol. Pathol.* 91 (2011) 419-428.
31. S. Ghoshal et al., Cyclooxygenase-2 deficiency attenuates adipose tissue differentiation and inflammation in mice, *J. Biol. Chem.* 286 (2011) 889-898.
32. H.R. Davis et al., Deficiency of Niemann-Pick C1 Like 1 prevents atherosclerosis in ApoE<sup>-/-</sup> mice, *Arterioscler. Thromb. Vasc. Biol.* 27 (2007) 841-849.
33. K.R. Feingold and C. Grunfeld, Cholesterol Lowering Drugs (2016) in: L.J. De Groot et al., (Eds.), *South Dartmouth*, 2000, PMID: 27809434.
34. J.L. Stöger et al., Deleting myeloid IL-10 receptor signalling attenuates atherosclerosis in LDLR<sup>-/-</sup> mice by altering intestinal cholesterol fluxes, *Thromb. Haemost.* 116 (2016) 565-577.
35. P.A. Alphonse and P.J. Hones, Revisiting human cholesterol synthesis and absorption: the reciprocity paradigm and its key regulators, *Lipids* 51 (2016) 519-536.
36. M.L. Jones et al., The human microbiome and bile acid metabolism: dysbiosis, dysmetabolism, disease and intervention, *Expert Opin. Biol. Ther.* 14 (2014) 467-482.
37. E.L. Gautier et al., Conventional Dendritic Cells at the Crossroads Between Immunity and Cholesterol Homeostasis in Atherosclerosis, *Circulation* 119 (2009) 2367-2375.
38. H.D. Manthey et al., Dendritic cells in atherosclerosis: Functions in immune regulation and beyond, *Thromb. Haemost.* 106 (2011) 772-778.
39. D. Milatovic et al., Protective effects of antioxidants and anti-inflammatory agents against manganese-induced oxidative damage and neuronal injury, *Toxicol. Appl. Pharmacol.* 256 (2011) 219-226.
40. P. Otero et al., Development of atherosclerosis in the diabetic BALB/c mice. Prevention with vitamin E administration, *Atherosclerosis* 182 (2005) 259-265.
41. I. Jialal and C.J. Fuller, Effect of vitamin E, vitamin C and beta-carotene on LDL oxidation and atherosclerosis, *Can. J. Cardiol.* 11 (1995) G97-G103.



42. T. Kita et al., Probucol prevents the progression of atherosclerosis in Watanabe heritable hyperlipidemic rabbit, an animal model for familial hypercholesterolemia, Proc. Natl. Acad. Sci. USA 84 (1987) 5928-5931.
43. P.M. Kris-Etherton, et al., Antioxidant vitamin supplements and cardiovascular disease, Circulation, 110 (2004) 637-641.
44. R. Stocker and J.F. Keaney Jr, Role of oxidative modifications in atherosclerosis, Physiol. Rev. 84 (2004) 1381-1478.

## 9. Figure legends

### Figure 1: Protective effect of D-PUFAs on LPO.

A, D-PUFAs inhibit the rate-limiting step of ROS-driven abstraction off a bis-allylic site. B, ROS-driven hydrogen abstraction off a bis-allylic site generates resonance-stabilized free radicals (C), which quickly react with abundant molecular oxygen to form lipid peroxy radicals (D). These newly formed ROS species (L-OO•) abstract hydrogen off a neighbouring PUFA molecule (turning themselves into lipid peroxides LOOH (E)), thus sustaining the chain by reacting with another molecule of B. LOOH (E), which have greater volume compared to non-oxidized lipids, further decompose through multiple pathways (particularly upon a prior enzymatic elongation/extension, not shown here, into higher PUFAs such as arachidonic acid, eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA)), into a smorgasbord of species (F) such as isoprostanes (e.g. F<sub>2</sub>-isoprostanes), reactive carbonyls, for instance 4-hydroxynonenal (4-HNE; a), 4-hydroxyhexenal (4-HHE; b), malondialdehyde (MDA; c), acrylic aldehyde (acrolein, AA; d), oxalic aldehyde (OA; e), 3-methylglutaric aldehyde (MGA; f). The chain is eventually terminated by a chain-terminating antioxidant or homologous recombination (not shown). The ethyl esters of D2-Linoleic acid (G) and D4-Linolenic acid (H) used in this study.

### Figure 2: D-PUFAs reduce lipid peroxidation products.

Lipid peroxidation products F<sub>2</sub>-isoprostanes (F<sub>2</sub>-IsoPs) and prostaglandin F<sub>2</sub>α (PGF<sub>2</sub>α) were determined in liver (A) and plasma (B) of *APOE\*3-Leiden.CETP* mice fed a D-/H-PUFA-containing WTD (until t=0 without cholesterol, thereafter containing 0.15% cholesterol, w/w). Values represent means ± SEM. (A: n=17-19 per group; B: n=8-10 pools of 2 mice per group). \**p*<0.05, \*\*\**p*<0.001.

### Figure 3: D-PUFAs reduce body fat mass, while increasing food intake.

Body weight gain (A), body fat mass gain (B) and body lean mass gain (C) were determined at the indicated time points in *APOE\*3-Leiden.CETP* mice fed a D-/H-PUFA-containing WTD (until t=0 without cholesterol, thereafter containing 0.15% cholesterol, w/w). Gonadal WAT (gWAT) fat pad weight was determined at the end of the study (D). Food intake was monitored during the study (E). Values represent means ± SEM. (n= 18-20 per group). \**p*<0.05, \*\*\**p*<0.001.

### Figure 4: D-PUFAs lower plasma cholesterol levels and alter hepatic cholesterol homeostasis.

Plasma triglyceride (TG; A) and total cholesterol (TC; B) were determined at the indicated time points in *APOE\*3-Leiden.CETP* mice fed a D-/H-PUFA-containing WTD (until t=0 without cholesterol, thereafter containing 0.15% cholesterol, w/w). The plasma total cholesterol exposure during the study was calculated (C). At the end of the study plasma non-HDL-cholesterol (non-HDL-C) and HDL-C were determined (D). In addition, hepatic cholesterol content (E) and hepatic markers of intestinal cholesterol absorption (F), cholesterol synthesis (G) and cholesterol breakdown (H) were measured. Values represent means ± SEM. (n= 18-20 per group). \**p*<0.05, \*\**p*<0.01, \*\*\**p*<0.001.

### Figure 5: D-PUFAs reduce atherosclerotic lesion development.

Slides of the valve area of the aortic root of *APOE\*3-Leiden.CETP* mice fed a D-/H-PUFA-containing WTD were stained with haematoxylin-phloxine-saffron and representative pictures are shown (A). Scale bar indicates 200 μm. Lesion area as a function of distance was assessed per mouse, starting from the appearance of open aortic valve leaflets (B). The mean

atherosclerotic lesion area was determined from the four cross-sections from **B (C)**. Values represent means  $\pm$  SEM. (n= 17-20 per group). \* $p$ <0.05, \*\* $p$ <0.01.

## Review report

We thank the reviewers for the positive words and their valuable suggestions for improvement. The comments are addressed as described below.

### Comments Reviewer #1:

Berbee and colleagues investigated the potential utility of deuterium-labelled polyunsaturated fatty acids (D-PUFAs) as antioxidants, antiatherosclerotic and lipid-lowering drugs in an established model of human-like lipoprotein metabolism. The main finding of this multi-centre study is that D-PUFAs can remarkably reduce lipid peroxidation and plasma cholesterol and thus atherosclerosis. In principle, treatment with D-PUFAs seems to be a promising new strategy against atherosclerosis.

The paper is well-written in all of its parts. Yet, the M & M should contain more information. This reviewer discusses the paper and makes some suggestions as follows below.

1. If there are no space-limitations by the journal, the title should involve the words "Deuterium" and "mice".

Authors' reply:

We adjusted the title of the manuscript to read: "*Deuterium-reinforced polyunsaturated fatty acids protect against atherosclerosis by lowering lipid peroxidation and hypercholesterolemia*". For reasons of simplicity we chose not to include the word 'mice'.

2. Introduction: provide the natural abundance of D.

Authors' reply:

We now included the natural abundance of deuterium in the following sentence of the Introduction section:

*"Deuterium is a stable hydrogen isotope that has natural abundance (150 ppm in ocean water) and is accordingly recognized by living systems as a normal, natural sub-type of hydrogen."*

3. M & M: please provide here the chemical and isotopic purity of the D-PUFAs. In addition, the authors should report the LPO biomarkers and the analytical methods used. Also, please report here how D contents were calculated.

Authors' reply:

Due to space limitations we provide only a short summary of the mice and general set-up of the study in the main manuscript. In addition, all the methods used in the current manuscript, including the analyses of LPO biomarkers, are standard methods and as such have extensively been described in previous manuscripts. We chose to give only a short description of the methods in the Supplemental Materials and Methods of the current manuscript and refer to the previous manuscripts reporting these methods.

Specific points:

- We now provided the chemical and isotopic purity of the D-PUFAs in the Supplemental Materials and Methods section (2<sup>nd</sup> paragraph, in the middle) as follows:

*“Chemical and isotopic purity of the synthetic D-PUFAs was in excess of 97.5-98%. They were synthesized, purified and characterized as described previously in detail [2,3].”*

- The LPO biomarkers are already reported in the Supplemental Materials and Methods, similar for the analytical methods used and reference to more detailed explanation. To better clarify how PGF<sub>2</sub>α was quantified we adjusted this paragraph to read (see also the response to comments 4b):

*“.... The method for F<sub>2</sub>IsoP analysis using GC/negative ion chemical ionisation MS has previously been described in detail [5-7]. PGF<sub>2</sub>α (which is present in vivo as a free acid) is quantifiable in the same analytical run.”*

- We realize it was not clear how D contents were calculated. In short, we just assessed the total level of deuterium present in the tissue and calculated the percentage of D-PUFA incorporation based on correlations with previous studies.

An assessment of D-PUFA incorporation into tissues requires detailed measurement of various thoroughly separated PUFA/D-PUFA species, by LC/GCMS methods. These data can then be correlated with the total increase in deuterium count, as measured by irMS. Accordingly, we rely on our previous detailed PUFA analysis studies to assess the level of D-PUFA incorporation (for example reference 27, correlated with deuterium (‰) measurements in reference 20+27). This was recently also confirmed in patients with Friedreich's ataxia treated with D-PUFAs (manuscript in preparation), which correlated well with studies in mice. Therefore, we are confident that the level of D-PUFA incorporation is quite accurate (approx. 30%) in the current study and well above the biologically relevant value to terminate LPO (10-20%).

To further clarify this to the reader we added the following sentence at the end of the paragraph describing 'Deuterium incorporation' in the Supplemental Materials and Methods section:

*“.... with R being the ratio between the abundances of deuterium and hydrogen (R = D/H). D-PUFA incorporation was assessed by correlating the deuterium incorporation with previous detailed D-PUFA incorporation studies [2,4].”*

#### **4. Results**

- a) please specify "F2-isoprostanes" and "bound". Which F2-isoprostanes are measured by the method used? Does "bound" mean esterified to lipids? Did the authors hydrolyzed the lipids?**

Authors' reply:

Indeed 'bound' means esterified to lipids. To clarify this to the reader we adjusted the methods in the Supplemental Materials and Methods as below, and also clarified that F<sub>2</sub>-IsoPs in plasma are measured as the unesterified form:

*“...at Vanderbilt University Medical Center. Unesterified F<sub>2</sub>-IsoPs were measured in plasma samples while esterified F<sub>2</sub>-IsoPs were measured in liver samples. Esterified F<sub>2</sub>-IsoPs were measured as free compounds following base hydrolysis. The method...”*

In addition, we completely removed the term 'bound' from the manuscript to avoid confusion and also revised Fig.2, the legend of Fig.2 and paragraph 3.2 in the Results section accordingly.

- b) please state about the identity of analyzed/measured PGF<sub>2</sub>α. Did you measure free non-esterified PGF<sub>2</sub>α?**

Authors' reply:

The reviewer is correct that PGF<sub>2</sub>α was measured as the free acid, as the enzymatic product is only generated as a free acid. We clarified this to the reader by adding the following sentence in the Supplemental Materials and Methods:

*“PGF<sub>2</sub>α (which is present in vivo as a free acid) is quantifiable in the same analytical run.”*

- c) Please specify "non-HDL-C".**

Authors' reply:

The term 'non-HDL-C' indicates all cholesterol that is not in the HDL fraction. In the Supplemental Materials & Methods section "Plasma lipid and liver toxicity parameters" we indicate that *“Plasma non-HDL-C levels were calculated by extracting HDL-C from TC levels.”* Under fasted conditions this means that non-HDL-C levels equals VLDL-C + LDL-C levels.

To further clarify this to the reader we adjusted the last sentence of the first paragraph of section 3.4 to read: *“The reduced plasma TC level (-4.6 mM at the endpoint) was confined to both reduced plasma non-HDL-C (-4.0 mM; -28%; calculated as TC minus HDL-C) and reduced plasma HDL-C (-0.7 mM; -48%) levels (Fig. 4D).”*

- d) Fig. 1A: Please remove the "X" above the arrow. Another symbol should be used to indicate that this step is not entirely excluded but it is "inhibited".**

Authors' reply:

We adjusted Fig. 1A accordingly.

- e) Fig. 1F: Please check "decomposition to isoprostanes". Which isoprostanes are meant?**

Authors' reply:

Many isoprostanes can be formed from these PUFAs as they can also be elongated into higher PUFAs. To further clarify this to the reader we adjusted the text of the legend of Fig.1 to read:

*“....LOOH (E), which have greater volume compared to non-oxidized lipids (particularly upon a prior enzymatic elongation/extension, not shown here, into higher PUFAs such as arachidonic acid, eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), into a smorgasbord of species (F) such as isoprostanes (e.g. F<sub>2</sub>-isoprostanes), reactive carbonyls....”*

In addition, we adjusted Fig. 1F to read: *“decomposition of isoprostanes, such as F<sub>2</sub>-isoprostanes”.*

- f) Fig. 2: Please explicitly state which fraction F<sub>2</sub>-IsoPs and PGF<sub>2</sub>α were measured: free, esterified or total; avoid use of "bound".**

Authors' reply:

As also indicated in the response to comment 4a we now completely removed the word 'bound' from the manuscript. In Fig. 2 we now clearly indicated that esterified F<sub>2</sub>-IsoPs were determined in liver and 'unesterified' F<sub>2</sub>-IsoPs in plasma.

- g) To this reviewer it is not clear how the authors measured D-incorporation of the supplied D-PUFAs. Please address this issue in more detail. For instance, were the D-PUFAs measured esterified to lipids?**

Authors' reply:

Please see the response to comment 3.

In addition, we also clarified this to the reader by adjusting the text in paragraph 3.1 of the Results section to read:

*"The difference between the D-PUFA (6,982±217‰) and the H-PUFA (-57±5‰;  $p<0.001$ ) group corresponds to approx. 30% D-PUFA incorporation (i.e. D-PUFA fraction of total PUFA) based on previous studies [20,27]."*

## **5. Discussion**

- a) Do D-PUFAs inhibit growth? Please comment/discuss.**

Authors' reply:

We found no evidence that D-PUFAs inhibit growth. Lean mass (absolute and gain) was similar between the groups and also the D-PUFA-treated mice did not appear smaller, while the mice were treated for 16 weeks with D-PUFAs. We now discuss this issue by adding the following sentence in the 3<sup>rd</sup> paragraph of the Discussion section:

*"... even more profound. It is likely also not the result of reduced growth as lean mass was not different between the groups. The reduced body fat mass ...."*

- b) Please explain the meaning of "PGF<sub>2</sub>α (formed primarily by enzymatic LPO)" do you mean COX?**

Authors' reply:

Indeed we mean by COX enzymes. We now clarified this to the reader by adjusting this sentence in the Discussion section (2<sup>nd</sup> paragraph) to read:

*"Comparing levels of F<sub>2</sub>-IsoPs (formed primarily by non-enzymatic LPO) with those of PGF<sub>2</sub>α (formed primarily by enzymatic LPO, i.e. by COX enzymes) reveals that while non-enzymatic LPO is substantially down-regulated in the D-PUFA group, the enzymatic oxidation is much less affected (Fig. 2)."*

- c) Please provide Reference "11,11-D2-linoleic acid is efficiently converted to 13-D1-arachidonic acid".**

Authors' reply:

We apologize for this typing error. We adjusted it to read:

*"...11,11-D2-linoleic acid is efficiently converted to 13,13-D2-arachidonic acid."*

- d) Isotopically labelled arachidonic acid has been used for several decades in studies on the formation of eicosanoids including oxidative stress. D8-Arachidonic acid has been recently reported to be converted by recombinant COX**

**to D-labelled PGE2, MDA and hydroxy-heptadecatrienoic acid (PMID: 22982857 and PMID: 27789233). Please consider these issues in the Discussion.**

Authors' reply:

We do not think the mentioned published work is relevant to our study, because metabolic studies using D-labelled PUFAs utilize the mass-shifting deuteration at the hydrophobic end, which is not involved in non-enzymatic LPO. Moreover, based on preliminary data in adults, infants and even pregnant women, we know that D-labelled PUFAs are not toxic and well tolerated. Therefore, we decided not to include this issue in the Discussion.

- e) For the sake of completeness the authors should make a comment with respect to the putative costs of D-PUFAs when used as drugs.**

Authors' reply:

We believe this is beyond the scope of the current manuscript, and will be addressed elsewhere in due course.

For this reviewer's information, we have scaled up the synthesis of one of the studied D-PUFAs, D2-lin ethyl ester (currently in clinic in humans), to a multiple tens of kilograms. The current COG per gram is hovering around 10 \$/g, and should go down substantially with the further scale up. We have also developed a completely novel approach to D-PUFA synthesis (manuscript in preparation), which should slash the COG and make longer D-PUFAs available (AA, DHA, EPA).

## **Comments Reviewer #2:**

***This highly interesting manuscript presents results of a very original study of the supplementation with D-PUFAs on atherosclerosis development, lipid peroxidation and cholesterol metabolism in an animal model of atherosclerotic disease. The concept of the study is excellent and the experimentation is well performed. However, the discussion of these promising data is not always sufficiently deep and detailed. The authors should thoughtfully address this point as well as deal with several minor comments as listed below.***

### **Major points**

- 1. Discussion of the observed effects of D-PUFAs should be deepened:**

- 1.1. Mechanisms linking regulation of inflammation to cholesterol metabolism should be thoughtfully presented, including potential roles of microbiota and dendritic cells.**

Authors' reply:

Based on the comments of the reviewer we included some speculation on possible underlying mechanism(s). In the original version of the manuscript we choose to not speculate too much on this issue as the underlying mechanism(s) may be complex and possibly the result of multiple pathways affected by D-PUFAs. In addition, the mixed plasma lipid profile (reduced plasma total cholesterol but increased plasma TG) makes this issue even more complicated for speculations. We now clarified this issue to the reader and included some speculation on possible underlying mechanism(s) as follows in the Discussion section:



4<sup>th</sup> paragraph Discussion section:

*“.....and plasma non-HDL-C levels in hyperlipidemic mice [28,29]. Our results indicate that this is a cholesterol-specific effect and not the result of a general reduction in chylomicron production and secretion, as plasma TG levels are unaffected or even increased. Reports on modulation of oxidative stress or inflammation in relation to intestinal cholesterol absorption are scarce. Interestingly, Stöger et al. [34] recently reported an anti-inflammatory mouse model with reduced intestinal cholesterol absorption and plasma TC levels, but the underlying mechanism(s) remain obscure. As treatment with D-PUFAs affect many mediators and possibly thus many processes, the exact mechanism(s) underlying the reduced intestinal cholesterol absorption upon D-PUFA treatment may be complex. Potential mechanism(s) may include modulation of the gut microbiome and/or bile acid metabolism in the gut [35,36], and modulation of (local) immune cells [37,38]. For example, dendritic cells, which are present in the inner lining of the intestines, may play a key role. Antioxidative strategies protect dendritic cells against degeneration [39], and increasing the lifespan and immunogenicity of dendritic cells reduces plasma cholesterol levels (via an unexplored mechanism) [37]. Together, one could speculate that D-PUFAs improve the lifespan and immunogenicity of the dendritic cells in the intestines, thereby reducing dietary cholesterol absorption and reducing plasma cholesterol levels. However, more detailed studies are required.”*

5<sup>th</sup> paragraph Discussion section:

*“However, also **potential** local antioxidative effects of D-PUFAs may have influenced these hepatic pathways of cholesterol synthesis and cholesterol breakdown directly. As outlined above for the intestinal cholesterol absorption, D-PUFAs affect many mediators and possibly thus many processes, and the exact underlying mechanism(s) may be complex. Taken together, our findings indicate that D-PUFAs lower the hepatic cholesterol content by reducing the intestinal cholesterol absorption and possibly by additional (local) antioxidative effects, thereby ultimately reducing plasma non-HDL-C levels.”*

## **1.2. Direct effects of D-PUFAs mediated by diminished LPO and/or reduced local inflammation require more detailed analysis.**

Authors' reply:

We agree with the reviewer that more detailed analyses should be performed to make strong conclusions on these issues and therefore only speculated on possible (in)direct and local effects. To meet the comment of the reviewer we further down-tuned several statements/speculations in the Discussion section as indicated below:

- 2<sup>nd</sup> paragraph: “... D-PUFAs thus have anti-atherogenic potential in hypercholesterolemic mice by reducing **systemic, and likely local**, non-enzymatic oxidative stress.”
- 5<sup>th</sup> paragraph: “However, also **potential** local antioxidative effects of D-PUFAs may have influenced these hepatic pathways of cholesterol synthesis and cholesterol breakdown directly.”
- 6<sup>th</sup> paragraph: “Finally, D-PUFA treatment reduced atherosclerotic lesion formation, which is in line with the reduced **systemic** oxidative and inflammatory status and reduced plasma non-HDL-C levels.”

- Final paragraph: “In conclusion, our data demonstrate that D-PUFAs limit body weight gain, improve cholesterol handling and reduce the development of atherosclerotic lesions by reducing **systemic, and likely** local, LPO and by reducing plasma non-HDL-C levels.”

### **1.3. Effects of D-PUFAs on body fat metabolism are highly interesting and their potential mechanisms should be suggested.**

Authors' reply:

We agree that the effect of D-PUFAs on body fat metabolism are highly interesting, however we did not want to speculate too much on this issue for the same reasons as described in the response to comment 1.1. However, based on this comment we now modified the text in the 3<sup>rd</sup> paragraph of the Discussion section to read:

*“The reduced body fat mass is in line with previous studies showing that reducing oxidative stress reduces obesity [29-30], but studies on potential underlying mechanism(s) are scarce. Therefore, the main mechanism(s) underlying the D-PUFA-mediated reduced fat mass gain remains to be identified, but may include reduced adipocyte differentiation as was observed in COX-2-deficient mice [31]. Overall .....”*

### **1.4. Finally, applicability of D-PUFA supplementation to humans should be developed in a separate paragraph.**

Authors' reply:

The D-PUFAs described in the current manuscript are currently in clinical trials in human neurological disease patients. The results will be reported in due course.

#### **Minor points**

- 1. Lipid peroxidation represents one of several pathways of LDL oxidation (Introduction, paragraph 2). It cannot be stated that "the oxidation of LDL is a non-enzymatic LPO chain process". One-electron and two-electron oxidants need to be introduced in this regard.**

Authors' reply:

We fully agree with the reviewer that there are other oxidation pathways that involve PUFAs. D-PUFAs are resistant to hydrogen abstraction off bis-allylic sites, but not to 2e-oxidants such as, for instance, singlet oxygen or HOCl. However, two-electron oxidation does not necessarily initiate the chain (*i.e.* it is stoichiometric), while the chain process may have the most damaging consequences, due to uncontrolled multiplication as well as a set of resulting toxic products particularly relevant to atherosclerosis pathology, such as 4-hydroxy-2-nonenal (HNE). There are enzymatic mechanisms that can initiate the chain reaction, in addition to non-enzymatic processes that can either initiate the chain, or oxidatively damage PUFAs in a stoichiometric, non-chain format. This intricate network of oxidative pathways is of great interest to us with regards the applicability of D-PUFAs to mitigating pathologies associated with lipid damage. We have, and are continuing, to extensively investigate these mechanistic aspects of D-PUFA action. However, we believe zooming in on such detail here would be outside of the scope of the current paper. We modified the text in the 2<sup>nd</sup> paragraph of the Introduction section as per below to reflect these considerations:

*“.... in the pathogenesis of atherosclerosis [4]. Polyunsaturated fatty acids (PUFAs) can get oxidized through either enzymatic or non-enzymatic pathways. Non-enzymatic damage can be initiated by both 2-electron- and 1-electron-oxidants, through an addition to a double bond, or, more typically (mostly for 1-electron-oxidants) by H-atom*

*abstraction off a bis-allylic methylene group. Once the radical is formed, the ensuing chain reaction of lipid peroxidation (LPO) multiplies the destruction. A smorgasbord of the downstream products of LPO include toxic carbonyls which further exacerbate the atherosclerosis-related damage, through the modification of lipids [5] and apoB within LDL [6] (Figure 1). Aldehyde-modified..."*

- 2. Recent publications need to be referred to when discussing the role of antioxidants in CV disease (Introduction, paragraph 3).**

Authors' reply:

We have updated the references in this paragraph by including 2 recent reviews.

- 3. Reference to support the description of the "kinetic isotope effect" is required (Introduction).**

Authors' reply:

We now included a reference to support the "kinetic isotope effect".

- 4. What is "sterol oxidative stress"? This should rather be reworded.**

Authors' reply:

We apologize for this typing error and have deleted the word 'sterol' in the final paragraph of the Introduction section.

- 5. Supplemental Table 1: the abbreviation gm% requires explanation.**

Authors' reply:

We adjusted this in Supplemental Table 1 to read "gram%".

- 6. More details regarding chromatographic approaches should be provided (primarily use of internal standards; see Supplemental Methods).**

Authors' reply:

As also indicated in the response to the next comment (#7), all our methods used in the current manuscript are standard methods. The analyses of hepatic cholestanol and sterols have extensively been described in other manuscripts, such as Lütjohann D. *et al.* 2004 Steroids (reference 8 in Supplemental Methods) and more recently in Kolsch H. *et al.* 2010 Biochim. Biophys. Acta (now included as reference 9 in Supplemental Methods). As we only used standard methods we chose to give only a short description of the methods and refer to the manuscripts that extensively describe these methods.

- 7. Non-standard methods need to be briefly presented in the main manuscript.**

Authors' reply:

All methods used in the current manuscript are standard methods. As we are limited in word count, we only provide a short summary of the mice and the set-up of the study in the main manuscript.

- 8. Results, section 3.1: calculations of D-PUFA incorporation and units employed require better explanation. This methodology should be included in the main manuscript (see the comment above).**

Authors' reply:

In short, we just assessed the total level of deuterium present in the tissue and calculated the percentage of D-PUFA incorporation based on correlations with previous studies.

An assessment of D-PUFA incorporation into tissues requires detailed measurement of various thoroughly separated PUFA/D-PUFA species, by LC/GCMS methods. These data can then be correlated with the total increase in deuterium count, as measured by irMS. Accordingly, we rely on our previous detailed PUFA analysis studies to assess the level of D-PUFA incorporation (for example reference 27, correlated with deuterium (‰) measurements in reference 20+27). This was recently also confirmed in patients with Friedreich's ataxia treated with D-PUFAs (manuscript in preparation), which correlated well with studies in mice. Therefore, we are confident that the level of D-PUFA incorporation is quite accurate (approx. 30%) in the current study and well above the biologically relevant value to terminate LPO (10-20%).

To further clarify this to the reader we added the following sentence at the end of the paragraph describing 'Deuterium incorporation' in the Supplemental Materials and Methods section:

*"... with R being the ratio between the abundances of deuterium and hydrogen ( $R = D/H$ ). D-PUFA incorporation was assessed by correlating the deuterium incorporation with previous detailed D-PUFA incorporation studies [2,4]."*

In addition, we clarified this to the reader by adjusting the text in paragraph 3.1 of the Results section to read:

*"The difference between the D-PUFA ( $6,982 \pm 217\%$ ) and the H-PUFA ( $-57 \pm 5\%$ ;  $p < 0.001$ ) group corresponds to approx. 30% D-PUFA incorporation (i.e. D-PUFA fraction of total PUFA) based on previous studies [20,27]."*

- 9. Results: Were bound F2-isoprostanes only determined in the liver and not in plasma (Figure 2)?**

Authors' reply:

Based on comments of both reviewers we completely removed the word 'bound' from the manuscript. We now clarify to the reader that 'esterified F<sub>2</sub>-IsoPs' (previously 'bound F<sub>2</sub>-IsoPs') were measured in liver samples, whereas 'unesterified F<sub>2</sub>-IsoPs' were measured in plasma samples. We revised this paragraph in the Supplemental Materials and Methods section as follows:

*"...at Vanderbilt University Medical Center. Unesterified F<sub>2</sub>-IsoPs were measured in plasma samples while esterified F<sub>2</sub>-IsoPs were measured in liver samples. Esterified F<sub>2</sub>-IsoPs were measured as free compounds following base hydrolysis. The method..."*

In addition, we revised Fig. 2 accordingly. The term 'bound' was also removed from paragraph 3.2 in the Results section and the legend of Fig.2.

- 10. Discussion, paragraph 4; the first sentence does not make sense and requires revision.**

Authors' reply:

We revised this sentence to read:

*“During cholesterol-feeding, D-PUFAs consistently reduced fasted plasma TC levels. This reduction was mainly attributed to reduced non-HDL-C levels and was also accompanied by a reduced hepatic cholesterol content at the end of the study. In-depth analyses of hepatic cholesterol metabolism ....”*

- 11. Discussion: what is "general and enzymatic inhibition of LPO" (pp. 9-10)? This terminology should be either clearly defined or revised.**

Authors' reply:

We modified this sentence in to read:

*“We propose that specific inhibition of non-enzymatic LPO, e.g. by D-PUFAs, may prove to be beneficial when targeting atherosclerosis and this process is likely superior to general antioxidant-based inhibition or employing nonsteroidal anti-inflammatory drugs (NSAIDs).”*

- 12. When discussing plausible role of LDL oxidation in atherogenesis, the authors should refer to the excellent review of Stocker and Keany (ref. 34) as well as to the response-to-retention model of atherosclerosis developed by Tabas and Williams.**

Authors' reply:

Although we agree with the reviewer that this is an important concept, we feel that it does not add to the focus and message of this paragraph in the Discussion section. However, to recognize the importance of this response-to-retention model we modified the 1<sup>st</sup> sentence of the 2<sup>nd</sup> paragraph in the Introduction section as follows:

*“Increased retention of low-density lipoprotein (LDL) in the vessel wall and subsequent oxidative modification is a crucial step in the pathogenesis of atherosclerosis [4].”*

**\*Statement of Originality**

Leiden, April 14, 2017

Dear Editor,

This manuscript entitled “Isotope-reinforced polyunsaturated fatty acids protect against atherosclerosis by lowering lipid peroxidation and hypercholesterolemia”, including supplemental files contains original research which has not been previously reported or published. The manuscript is not under consideration elsewhere, and will not be submitted elsewhere while under review by ‘Atherosclerosis’, and after possible acceptance for publication.

Sincerely,

Jimmy F.P. Berbée

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Leiden, June 2, 2017

Dear Dr. Laurent Yvan-Charvet,

Thank you very much for the evaluation of our manuscript ATH-D-17-00433, entitled "*Isotope-reinforced polyunsaturated fatty acids protect against atherosclerosis by lowering lipid peroxidation and hypercholesterolemia*".

We thank the two reviewers for their helpful comments, which we used to improve the manuscript. Based on the reviewers' comments, we now among others further clarified some measurements and statements, deepened the Discussion section and revised Fig. 1 and 2. We chose to limit the adjustments to the (Supplemental) Materials and Methods section as we only use standard methods which have been described in detail in the referenced manuscripts. We also indicated this in the response to the reviewers. If the editor still insists on extending the (Supplemental) Materials and Methods section we will do so.

Please find enclosed the revised manuscript (with the changes indicated in red) as well as a letter detailing the changes made in accordance with the comments of the reviewers. Changes in the Supplemental Materials and Methods, Supplemental Table 1 and Figures have not been indicated in red.

All authors have read the revised manuscript and approved submission. We hope our manuscript is now acceptable for publication in *Atherosclerosis*.

Looking forward to hearing from you,

On behalf of all authors,

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**\*Conflict of interest form**

Leiden, April 14, 2017

Dear Editor,

Lex H.T. van der Ploeg and Mikhail S. Shchepinov receive compensation from and hold stock in Retrotope, Inc.

Financial support and D-PUFA reagents were provided by Retrotope, Inc.

The other authors have no conflicts to declare.

Sincerely,

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Figure(s)

Figures - Berbée *et al.*  
D-PUFAs reduce atherosclerosis

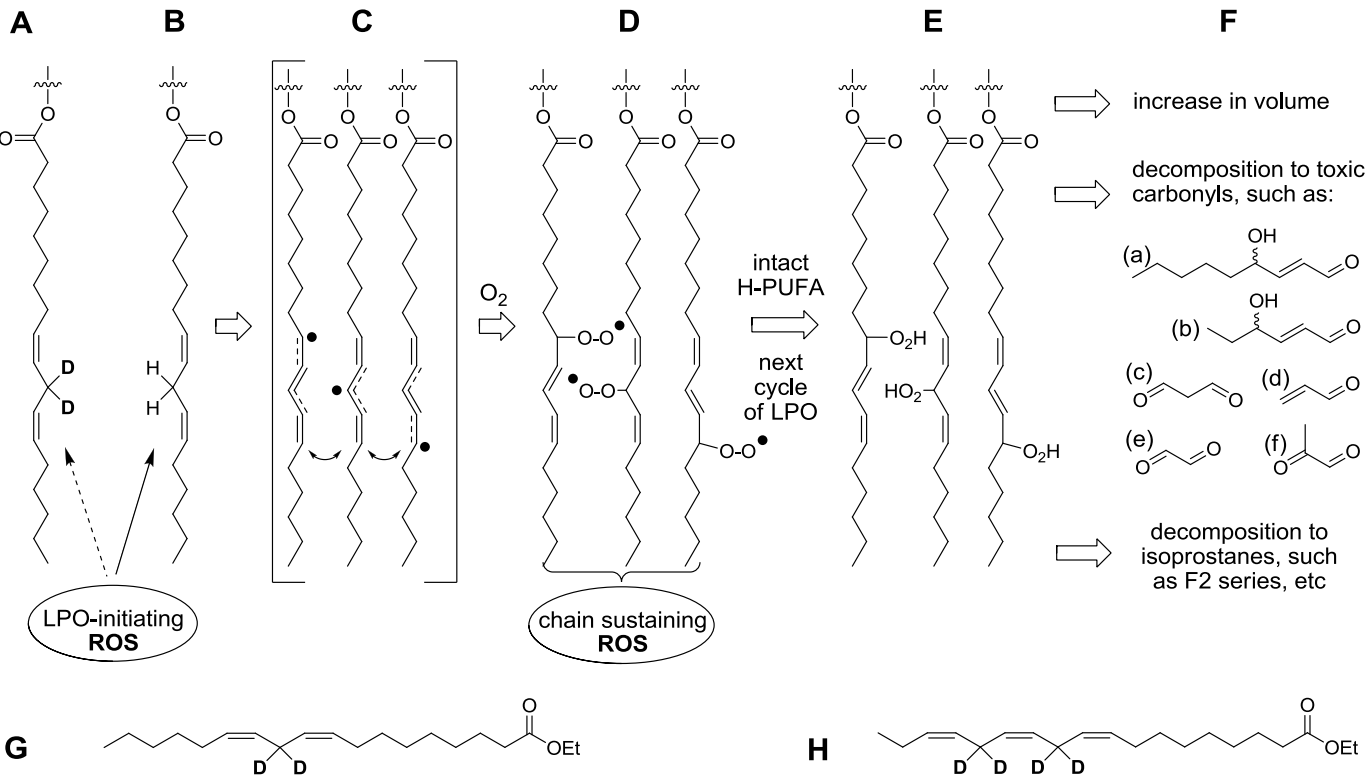
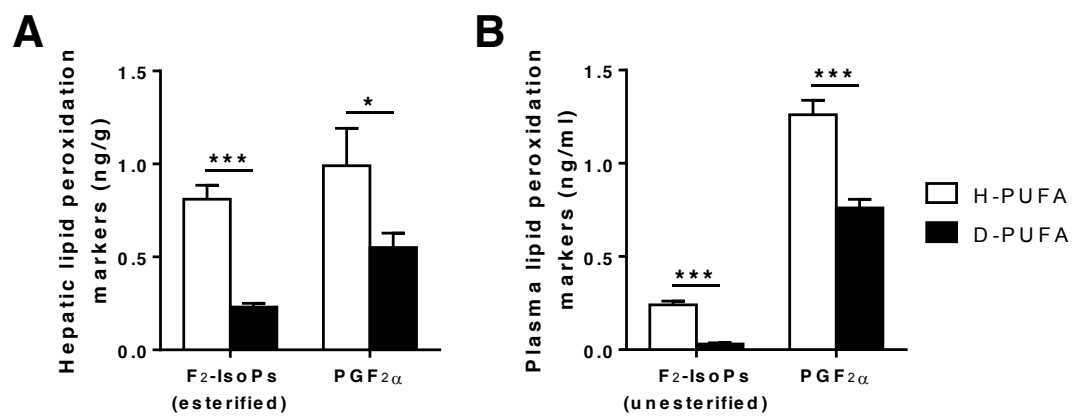
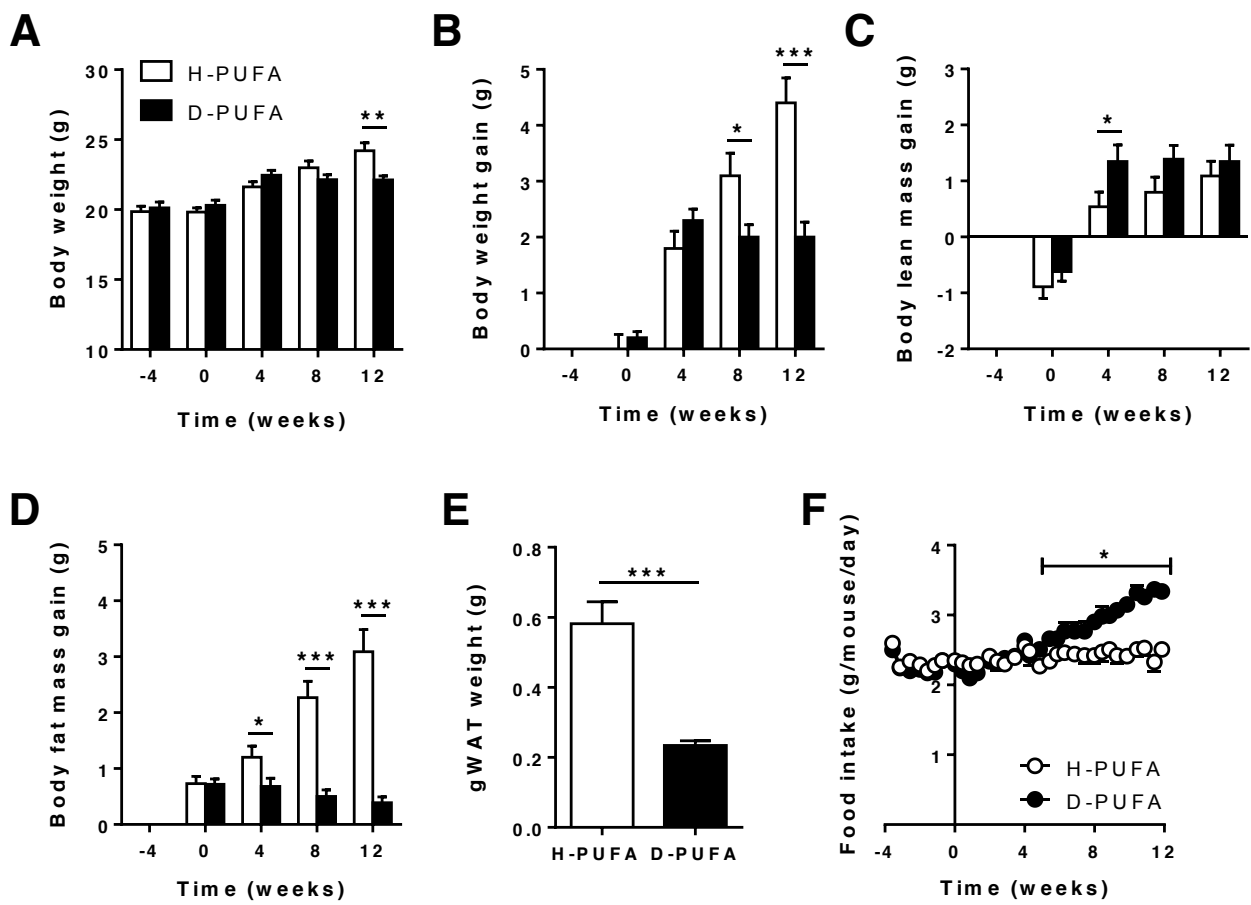


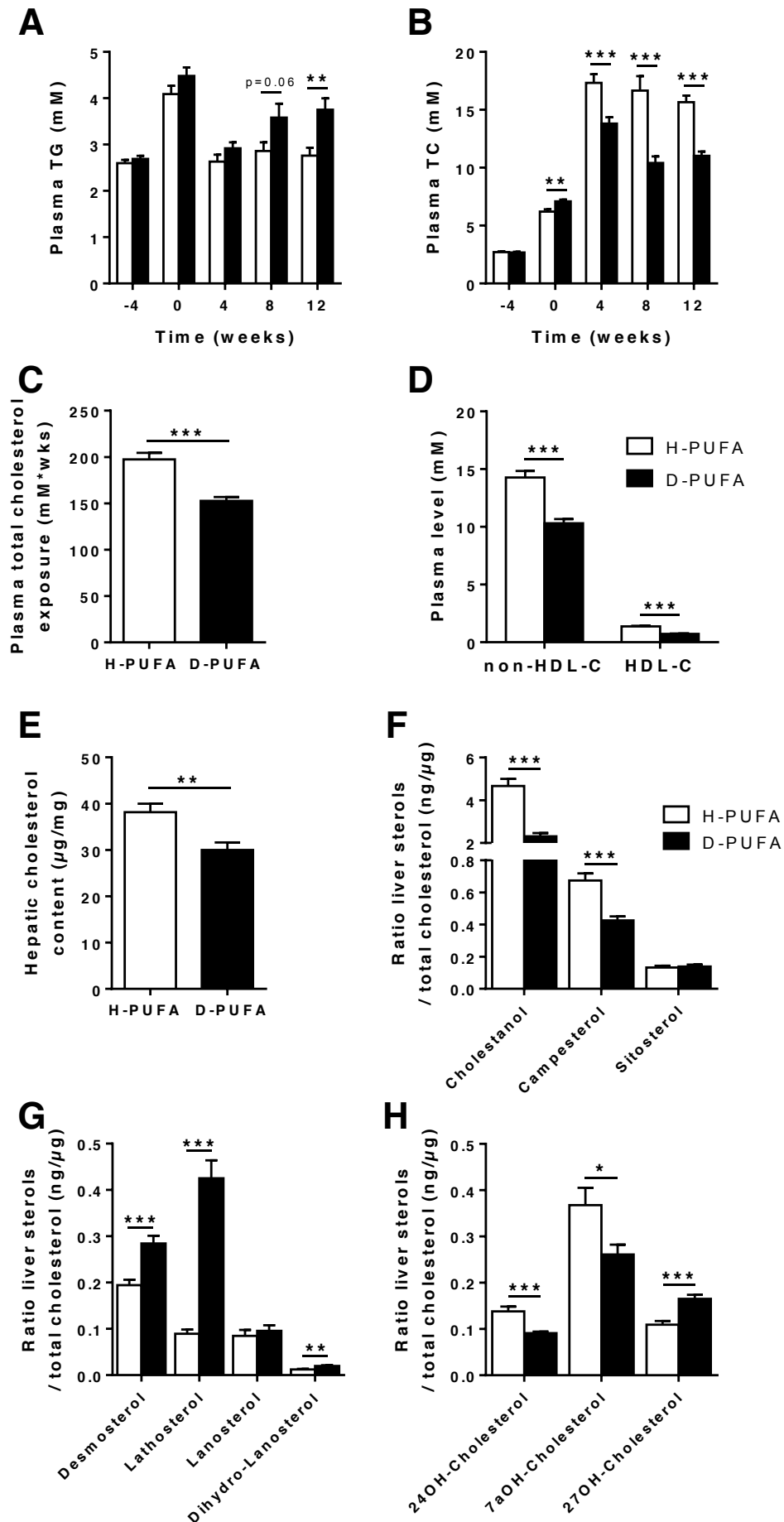
Figure 1



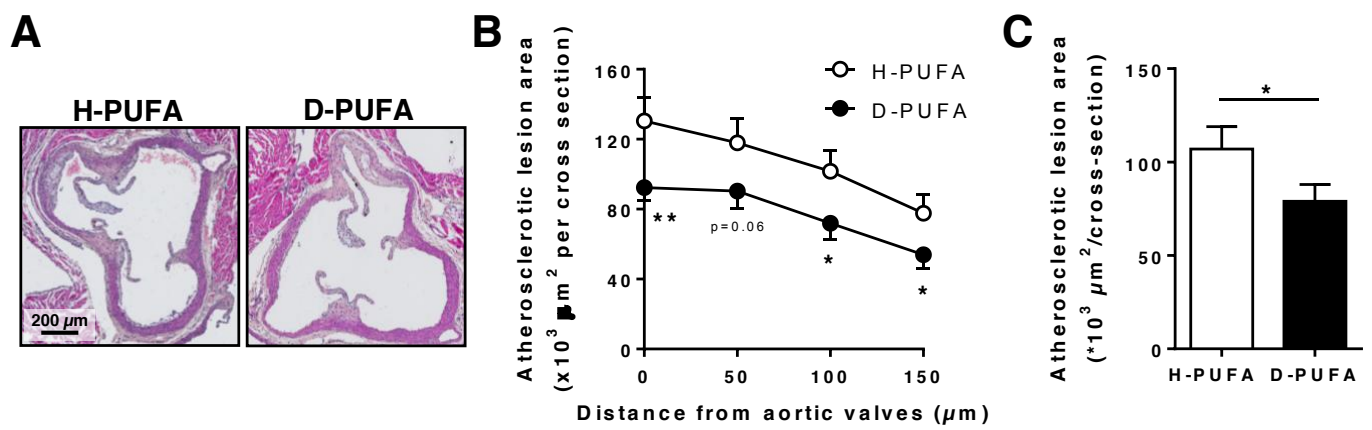
**Figure 2**



**Figure 3**



**Figure 4**



**Figure 5**

**Supplementary Material for online publication only**

[Click here to download Supplementary Material for online publication only: Revised Supplemental Mat&Met Berbe et al. - D-PUF.](#)

**Supplementary Material for online publication only**  
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## \*Atherosclerosis style guide checklist

### Atherosclerosis style guide checklist

*Atherosclerosis* applies format guidelines to all accepted papers, with the aim of improving their readability.

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Please find below a questionnaire to guide authors to comply with the formatting requirements for revised submissions. For more detailed information, visit [our website](#).

**Please note that when you answer “No” to a question, editing of your manuscript is required before submission to *Atherosclerosis*.**

#### Manuscript structure and style

**Does your manuscript contain all the below essential elements, in this order?**

**Yes    No**

(please stick to the headers as indicated below)

- Title
- Authors, Affiliations, Contact Information
- Abstract in the Atherosclerosis format (*Background and aims, Methods, Results, Conclusions*)
- Introduction
- Materials and methods (or Patients and methods)
- Results
- Discussion
- Conflict of interest (mandatory)
- Financial support (if applicable)
- Author contributions (mandatory)
- Acknowledgements (if applicable)
- References
- Figures and Tables (with legends in the suitable style)

#### Abstract style

**Is the Abstract structured in the below sections?**

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- *Background and aims*
- *Methods*
- *Results*
- *Conclusions*

#### Figure and table legends

**Are figure and table legends formatted as described below?**

**Yes    No**

Each figure and table legend should have a brief overarching title that describes the entire figure without citing specific panels, followed by a description of each panel, and all symbols used.

If a figure or table contains multiple panels, the letter describing each panel should be capitalized and surrounded by parenthesis: i.e. (A)(B)(C)(D).

Please make sure to apply the formatting requirements to figures and tables where necessary (e.g. style of *p* values, gene and protein nomenclature).

#### Footnotes to tables

**Are footnotes to tables formatted as described below?**

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Footnotes to tables should be listed with superscript lowercase letters, beginning with “<sup>a</sup>.”  
Footnotes must not be listed with numbers or symbols.

#### Abbreviations

**Are abbreviations defined when first used in the text?**

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Use of abbreviations should be kept at a minimum.



### Units

Are units expressed following the international system of units (SI)?

Yes No

If other units are mentioned, please provide conversion factors into SI units.

### DNA and protein sequences

Are gene names italicized?

Yes No

Gene names should be italicized; protein products of the loci are not italicized.

For murine models, the gene and protein names are lowercase except for the first letter.  
(e.g., gene: *Abcb4*; protein: Abcb4)

For humans, the whole gene name is capitalized.  
(e.g., gene: *ABCB4*; protein ABCB4)

### Mouse strains and cell lines

Are knock-out or transgenic mouse strains and cell lines italicized and the symbol superscripted? Yes No

(e.g. *ob/ob* , *p53<sup>+/+</sup>* , *p53<sup>-/-</sup>*)

### p values

Are p values consistently formatted according to the below style throughout the manuscript (including figures and tables)?

Yes No

$p < X$

$p > X$

$p = X$

### Language

Is your manuscript written in good English?

Yes No

Please make sure that you consistently use either American or British English, but not a mixture of them.

Please make sure that words are written consistently in the same way throughout the manuscript.

e.g. non-significant or nonsignificant

e.g. down-regulation or downregulation

### Artwork

Have you submitted high-resolution versions of your original artwork?

Yes No

Please make sure to use uniform lettering and sizing in your original artwork, including letters to indicate panels, consistently throughout all figures.