RESEARCH ARTICLE

Nutritional depletion in n-3 PUFA in apoE knock-out mice: A new model of endothelial dysfunction associated with fatty liver disease

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Scope: Western diets are characterized by low intake of n-3 PUFA compensated by constant amounts of n-6 PUFA. Reduced intake of n-3 PUFA is associated with increased cardiovascular risk, as observed in nonalcoholic fatty liver disease patients. The study aimed to evaluating the impact of dietary n-3 PUFA depletion on endothelial function, an early event of cardiovascular diseases.

Methods and results: C57Bl/6J or apolipoprotein E knock-out (apoE⁻/⁻) were fed control (CT) or n-3 PUFA-depleted diets (DEF) for 12 wks. Mice fed n-3 DEF diet developed a hepatic steatosis, linked to changes in hepatic expression of genes controlled by Sterol Regulatory Element Binding Protein-1 and -2. Vascular function was assessed on second- and third-order mesenteric arteries and n-3 PUFA-depleted apoE⁻/⁻ mice presented endothelial dysfunction characterized by decreased vasorelaxation in response of acetylcholine. The presence of a nitric oxide synthase (NOS) inhibitor blunted the relaxation in each groups and heme-nitrosylated hemoglobin blood (Hb-NO) level was significantly lower in n-3 PUFA-depleted apoE⁻/⁻ mice.

Conclusion: Twelve weeks of n-3 DEF diet promote steatosis and accelerate the process of endothelial dysfunction in apoE⁻/⁻ mice by a mechanism involving the NOS/NO pathway. We propose n-3 PUFA-depleted apoE⁻/⁻ mice as a new model to study endothelial dysfunction related to hepatic steatosis independently of obesity.

Keywords: apoE knock-out mice / Endothelial dysfunction / Liver / n-3 PUFA / Nitric oxide

Additional supporting information may be found in the online version of this article at the publisher’s web-site

1 Introduction

The burden of cardiovascular diseases is high and still growing. Lifestyle-related risk factors, among which insufficient intake of key nutrients and overconsumption of fat and energy, have the greatest impact on cardiovascular-related deaths [1–3].

Western diets are characterized by low dietary intake of n-3 PUFA compensated by constant amounts of n-6 PUFA [4, 5]. Poor dietary n-3 PUFA intake has consistently been
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Table 1. Composition of CT and DEF diets

<table>
<thead>
<tr>
<th>Composition (%)</th>
<th>CT diet (D08041805)</th>
<th>DEF diet (D08041806)</th>
</tr>
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<tr>
<td>Casein (%)</td>
<td>20</td>
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<tr>
<td>Total carbohydrates (%)</td>
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<tr>
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<td>Sucrose (%)</td>
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<td>13.2</td>
</tr>
<tr>
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<tr>
<td>Soybean oil (%)</td>
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</tr>
<tr>
<td>C16:0 (%)</td>
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<td></td>
</tr>
<tr>
<td>C18:0 (%)</td>
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<td></td>
</tr>
<tr>
<td>C20:0 (%)</td>
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</tr>
<tr>
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<tr>
<td>C18:2 n-6 (%)</td>
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</tr>
<tr>
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<td></td>
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<td>Sunflower oil (%)</td>
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<tr>
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</tr>
<tr>
<td>C20:0 (%)</td>
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<tr>
<td>C16:1 (%)</td>
<td>0.02</td>
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</tr>
<tr>
<td>C18:1 (%)</td>
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<tr>
<td>C18:2 n-6 (%)</td>
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<td></td>
</tr>
<tr>
<td>C18:3 n-3 (%)</td>
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<td>Mineral mix (%)</td>
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<td>3.5</td>
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<tr>
<td>Vitamin mix (%)</td>
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</tr>
<tr>
<td>kcal/g</td>
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</tbody>
</table>

Formulated by Research Diets. Parenthetical numbers indicate the manufacturer’s diet number.

2 Materials and methods

2.1 Animals and diets

Nine-wk-old male C57Bl/6J (WT, n = 11/diet) and apoE−/− (KO, n = 9/diet) mice (Charles River Laboratories, L’Arbresle, France) were housed two or three mice per cage with a 12 h light/dark cycle at 22°C, diet and water ad libitum.

Mice were fed a CT diet (D08041805, Research Diets, New Brunswick, NJ, USA) or n-3 PUFA-depleted diet (DEF) (D08041806, Research Diets) for 12 wks. The n-3 PUFA depletion was induced by replacing the soybean oil with sunflower oil. Compositions of both diets are described in Table 1. Body composition was assessed every 4 wks by using 7.5 MHz time-domain NMR (LF50 minispec, Bruker, Rheinstetten, Germany).

At the end of treatment, mice were anaesthetized in fed state (ketamine/xylazine i.p., 100 and 10 mg/kg of body weight, respectively). Venous blood was obtained by a puncture of the right ventricle, immediately frozen in heparinized calibrated tube (0.2 mL) in liquid nitrogen and analyzed by Electron Paramagnetic Resonance (EPR) spectroscopy for nitric oxide (NO) measurement. The remaining volume of venous blood was collected and centrifuged (3 min at 13 000 × g)
for storage. Liver was removed and immediately frozen in liquid nitrogen. The samples were stored at −80°C for further analysis.

The experiments were approved by and performed in accordance with the guidelines of the local ethics committee. Housing conditions were specified by the Belgian Law of May 29, 2013, regarding the protection of laboratory animals (agreement n° LA1230314).

2.2 Measurement of NO bioavailability by EPR

The level of circulating heme-nitrosylated hemoglobin (Hb-NO) was assayed in whole blood of mice from the EPR signal of 5-coordinate-α-Hb-NO as previously described [20]. The EPR spectra of whole blood were recorded using a Bruker EMXmicro spectrometer with following settings: microwave power, 20 mW; modulation amplitude, 0.7 mT; time constant, 80 ms; 5 scans, at temperature 77 K. The level of Hb-NO was quantified from hyperfine structure (g = 2.011; A = 16.8 G) of the EPR signal after subtraction of signal of protein-centered free radicals (g = 2.005; linewidth ~19 G) using Xenon software.

2.3 Measurement of contraction and relaxation

Second- and third-order mesenteric arteries were rapidly removed and carefully isolated from visceral adipose and connective tissues in ice-cold solution (Tyrode solution composition in mM: NaCl 128.3, KCl 4.5, NaHCO₃ 20.23, NaH₂PO₄ 0.35, glucose 11, CaCl₂ 1.35, MgSO₄ 1.05) gassed with carbogen. Artery segments of 2 mm in length were mounted in a wire myograph (model 610M and 620M, Danish Myo Technology A/S, Aarhus, Denmark). Arteries were maintained under zero force for 45 min at 37°C. Normalization of each artery was performed to calculate the effective transmural pressure. Arteries were set at a tension equivalent to that generated at 90% of the artery diameter at 100 mm Hg. All procedures were performed in absence and presence of indomethacin (indo; 0.01 mM). After 45 min of equilibration, arteries were challenged with a high KCl solution (KCl-tyrode solution composition in mM: NaCl 37.58, KCl 96.08, NaHCO₃ 20.23, NaH₂PO₄ 0.35, glucose 11, CaCl₂ 1.35, MgSO₄ 1.05) to assess the viability and the maximal contraction. Endothelium-dependent relaxation was evaluated by cumulative addition of acetylcholine (from 10⁻⁸ to 3.10⁻⁵ M) on precontracted arteries with a high KCl solution, in the presence or the absence of NO synthase (NOS) inhibitor Nω-Nitro-L-arginine methyl ester (L-NAME; 100 μM).

2.4 Blood biochemical analyses

Triglycerides, total CHOL and HDL-CHOL (HDLc) concentrations were measured in nonthawed plasma using kits (Diasys Diagnostic and Systems, Holzheim, Germany), as previously described [21]. Plasma concentrations of cytokines (IL-6, IL-10, IL-1β, IL-17A, TNF-α, and IFN-γ) and other analytes (sE-Selectin, sICAM-1, plasminogen activator inhibitor-1 Total, proMMP-9) were determined using two multiplex immunoassay kits (Bio-Plex Cytokine Assay from Bio-Rad and Milliplex Mouse CVD 1 from Merck Millipore, respectively). Both analyses were performed by Luminex® technology (BioplexH, Bio-Rad, Belgium).

2.5 Liver histological analysis

Frozen sections obtained from a fraction of the main liver lobe mounted in embedding medium (Tissue-tek, Sakura, The Netherlands) were sliced and stained with the oil red O, using 0.5% oil red O dissolved in propylene glycol for 10 min at 60°C. The sliced sections were then counterstained with a Hemalum solution and scanned with a Leica whole slide scanner SCN400. The size and distribution of the lipid droplets were determined by thresholding for the Oil Red O signal using the freely available imaging software ImageJ (version 1.48r, National Institutes of Health, Bethesda, MD, USA). At least five fields per animal were analyzed.

2.6 Real-time quantitative PCR

Total RNA was isolated from liver by TriPure reagent (Roche, Basel, Switzerland). cDNA was prepared by reverse transcription of 1 μg total RNA using Reverse Transcription System kit (Promega, Leiden, The Netherlands). Real-time qPCRs were performed with a StepOnePlus instrument and software (Applied Biosystems, Foster City, CA, USA) using Mesa Fast qPCR (Eurogentec, Seraing, Belgium) for detection. Ribosomal protein L19 (RPL19) RNA was chosen as housekeeping gene. Sequences of the primers are summarized in Supporting Information Table 1. All samples were run in duplicate in a single 96-well reaction plate and data were analyzed according to the 2⁻ΔΔCT method [22]. The identity and purity of the amplified product were checked through analysis of the melting curve carried out at the end of amplification.

2.7 Western blot

Cytoplasmic and nuclear proteins were extracted following manufacturer’s instruction (NE-PER®, Thermo Scientific, Waltham, MA, USA) from 50 mg of liver. Equal amount proteins were separated by 10% SDS/PAGE and transferred to nitrocellulose membrane, blocked in Tris-buffered saline tween-20 containing 5% nonfat dry milk for 1 h at room temperature. The membranes were incubated overnight

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Figure 1. Weekly and cumulative food intake, body composition, and body weight gain of WT CT, WT DEF, KO CT and KO DEF mice. Weekly food intake (A) was measured and cumulative food intake (B) was calculated on the basis of two or three mice per cage and three or four cages per groups. The body composition was assessed every 4 wks, and the results are expressed in gram of fat mass per mouse (C) and gram of lean mass per mouse (D). The body weight gain (D) represents the difference between the initial body weight and the 12 wks later body weight, expressed in grams. Each parameter was analyzed in WT mice fed a CT diet (WT CT, \( n = 11 \)), WT mice fed an \( n \)-3 DEF diet (WT DEF, \( n = 11 \)), apoE\(^{-/-}\) mice fed a CT diet (KO CT, \( n = 9 \)), and apoE\(^{-/-}\) mice fed an \( n \)-3 DEF diet (KO DEF, \( n = 9 \)). Data are expressed as the mean ± SEM. Data were analyzed by one-way or two-way ANOVA followed by multiple comparison tests: \(^*\) for KO CT versus WT CT, \(^\$\) for KO CT versus WT DEF, \(^#\) for KO DEF versus WT DEF.

at 4°C with the rabbit anti-SREBP-2 antibody (Abcam, Cambridge, UK) or the mouse anti-SREBP-1 antibody (Thermo Scientific) in Tris-buffered saline tween-20 containing 1% nonfat dry milk. Signals were revealed using ECL Western blotting substrates (SuperSignal West Pico Substrate, Thermo Scientific). TATA binding protein and beta-actin (both from Abcam) were used as loading CT. Gels are analyzed and quantified by ImageQuant TL instrument and software version 8.1 (GE Healthcare, Buckinghamshire, England).

2.8 Statistical analysis

Results are presented as mean ± SEM. After normality was checked, Dixon’s Q-test was performed to statistically reject outliers (95% confidence level). Statistical significance between groups was assessed by one-way analysis of variance (ANOVA) followed by Tukey’s multiple comparison tests. Grouped analyses were assessed by two-way ANOVA followed by Bonferroni’s multiple comparison tests. Data with superscript symbol (\(^*, \$\), or \(^#\)) were significantly different as indicated in all figure legends according to \( ^* \) \( p \leq 0.05 \), \( ^\$ \) \( p \leq 0.01 \), and \( ^\$\$\$ \) \( p \leq 0.001 \). Statistical analyses were performed using GraphPad Prism version 5.00 for windows.

3 Results

3.1 \( n \)-3 PUFA depletion in apoE\(^{-/-}\) mice does not modify anthropometric parameters

Food intake was measured weekly and there was no difference between WT mice fed either CT diet (WT CT) or \( n \)-3 DEF diet (WT DEF) throughout the 12 wks of treatment. This was confirmed by calculating the cumulative food intake (Fig. 1A and B). As previously reported [23], apoE\(^{-/-}\) mice fed a CT diet (KO CT) presented a slightly, but significantly, increased food intake from wk 1 to the end of the treatment, correlating with a significantly higher cumulative food intake compared to WT mice. In contrast, daily food intake of apoE\(^{-/-}\) mice fed an \( n \)-3 DEF diet (KO DEF) for 12 wks was similar to the one observed in WT mice (Fig. 1A and B).

Body composition was assessed every 4 wks. The fat mass was lower in apoE\(^{-/-}\) mice fed a normal diet versus their wild-type CTs and this remained different throughout the duration of the experiment. This was related to a higher lean mass in CT apoE-deficient animals. \( n \)-3 DEF diet did not modify the body composition of wild-type mice but it blunted the differences between genotypes in the evolution of lean mass (Fig. 1C and D). In terms of overall body weight gain, no significant difference was observed between groups after treatment (Fig. 1E).
3.2 \textit{n}-3 PUFA depletion promotes the development of hepatic steatosis

Histological analysis performed on frozen sections of the main lobe revealed a significantly larger lipid surface area in the liver of WT DEF and KO DEF mice, than in mice fed a CT diet, suggesting the development of hepatic steatosis associated to the 12 wks of \textit{n}-3 PUFA depletion (Fig. 2A and B). The average size of lipids droplets tended to be higher in WT DEF and KO DEF mice but this parameter did not reach statistical significance (Fig. 2C). The liver tissue weight was not modified by \textit{n}-3 PUFA depletion, whatever is the genetic background of the mice (Fig. 2D).

3.3 \textit{n}-3 PUFA-depleted mice display hepatic metabolic alterations associated with changes in SREBP-1c and SREBP-2

Sterol regulatory element binding proteins (SREBPs)-1c and -2 are key transcription factors regulating lipogenesis and cholesterologenesis, respectively. Interestingly, WT mice fed an \textit{n}-3 DEF diet showed an important increase in the expression of major targets of SREBP-1c (fatty acid synthase [FAS] and stearoyl-coA desaturase [SCD-1]) (Fig. 3A) and SREBP-2 (3-hydroxy-3-methylglutaryl coenzyme A reductase [Hmgcr] and LDLr) (Fig. 3B) compared to WT CT mice. KO DEF mice presented a similar gene upregulation compared to their own CT mice; this effect was significant for Hmgcr, SREBP-1c, FAS, SCD-1 (Fig. 3A and B). SREBP-1c and -2 are synthesized as inactive precursors embedded in the reticulum endoplasmic membrane and need to be enzymatically cleaved prior their translocation to the nucleus [24]. Therefore, we performed Western blot analysis in nuclear and cytoplasmic proteins homogenates to measure the mature/active versus precursor/inactive form of SREBP-1 and -2. Western blots analysis revealed a higher hepatic content of mature SREBP-1 (mSREBP-1) and -2 (mSREBP-2) in the liver of WT DEF, compared to WT CT mice. KO DEF mice also presented an increased content of mSREBP-2 compared to KO CT mice. Hepatic precursors SREBP-1 (pSREBP-1) and -2 (pSREBP-2) were not significantly different in the cytoplasm between groups (Fig. 3C). Our observations of a modulated expression of genes involved in fatty acid and CHOL synthesis could account for the reported increase in hepatic lipid storage observed in WT and KO mice fed an \textit{n}-3 DEF diet.

3.4 Mesenteric arteries from KO DEF mice present an endothelial dysfunction involving NOS/NO pathway

Mesenteric arteries segments were isolated from mice and mounted in a wire myograph. After normalization, resting parameters revealed no difference not only between the four groups concerning the basal tone, the mean arterial diameter but also the maximal contraction in response to KCl challenge (50 mM) (Fig. 4A–C). The presence of a nonselective cyclooxygenase inhibitor, indomethacin (indo), in the bathing medium did not significantly modulate the resting parameters (Fig. 4C). The endothelium-dependent relaxation was evaluated by cumulative addition of acetylcholine on
preconstricted arteries in the presence of a KCl-enriched solution. This evaluation was performed under three different conditions: alone, in the presence of indo, or in the presence of an NO synthase inhibitor (L-NAME) in order to evaluate the different components of the endothelium-dependent relaxation (total and NO versus prostacyclin-mediated relaxations). We observed that mesenteric arteries isolated from KO DEF mice relaxed significantly less in response to acetylcholine than vessels from WT DEF and WT CT mice, indicating the presence of endothelial dysfunction in KO DEF mice. In comparison, KO CT mice did not develop a significant endothelial dysfunction, compared to WT mice (Fig. 4D). Incubation of mesenteric arteries with indo did not considerably alter the result, and confirmed the reduced endothelial function of KO DEF arteries in response to muscarinic stimulation compared to WT CT vessels (Fig. 4E). Incubation with L-NAME completely blunted the relaxation suggesting that most of the relaxation in response to acetylcholine relies on the activation of NOS. Under these conditions, no differences between genotypes and/or treatments were observed (Fig. 4F). To ascertain the impact of n-3 PUFA deficiency and genotype on the NOS/NO pathway, we measured the level of circulating Hb-NO in venous blood by EPR. KO CT mice displayed a 40% decreased level of Hb-NO compared to WT CT mice, although this did not reach statistical significance. Feeding WT and KO mice with n-3 DEF diet further decreased circulating the Hb-NO levels (Fig. 4G). Circulating biomarkers of inflammatory related to cell adhesion molecules as sICAM-1, sE-Selectin, and pro-MMP-9 were not significantly influenced by diet or genotypes. Total plasminogen activator inhibitor-1 was significantly increased in KO CT and KO DEF mice, compared to WT CT mice (Supporting Information Fig. 1).

3.5 apoE deficiency leads to altered lipids profile without changing inflammatory state

Triglyceridemia was not affected by n-3 PUFA depletion in any genotypes (Table 2). As expected, KO mice presented a two-fold increase in total CHOL level in plasma due to the absence of apoE. This effect was even more marked in KO mice fed an n-3 DEF diet for 12 wks. Interestingly, n-3 PUFA depletion did not significantly affect total CHOL levels in WT mice, however, we observed a further reduction of HDLc in...
Figure 4. Resting parameters, contractile profile, endothelium-dependent relaxation in mesenteric microarteries and level of Hb-NO in venous blood. Basal tone (A), vessel diameter (B), and maximal contraction (C) in response of KCl (50 mM) challenge (in presence or in absence of cyclooxygenase inhibitor, indo) from second- and third-order mesenteric arteries from WT mice fed a CT diet (WT CT, n = 11), WT mice fed an n-3 DEF diet (WT DEF, n = 10–11), apoE⁻/⁻ mice fed a CT diet (KO CT, n = 7), and apoE⁻/⁻ mice fed an n-3 DEF diet (KO DEF, n = 7) for 12 wks. (D) Evaluation of endothelium-dependent relaxation by increasing doses of acetylcholine on preconstricted microarteries, (E) in presence of cyclooxygenase inhibitor (indo, 0.01 М), (F) and in presence of NOS inhibitor (L-NAME, 100 μM for 15 min). Level of Hb-NO in venous blood from WT mice fed a CT diet (WT CT, n = 7), WT mice fed an n-3 DEF diet (WT DEF, n = 11), apoE⁻/⁻ mice fed a CT diet (KO CT, n = 7), and apoE⁻/⁻ mice fed an n-3 DEF diet (KO DEF, n = 7) for 12 wks. Data are expressed as the mean ± SEM. Data were analyzed by one-way or two-way ANOVA followed by multiple comparison tests: * for WT CT versus KO DEF, # for WT DEF versus KO DEF, $ for WT CT versus WT DEF.

4 Discussion

Genetic deficiency in apolipoprotein E (apoE) in mice provokes an impairment of CHOL clearance, leading to hypercholesterolemia. Upon ageing, or earlier in life if animals are fed a high-fat diet, apoE-deficient mice develop impaired endothelium-dependent relaxation, an early marker of cardiovascular diseases and their consequences [25, 26]. Nutrition plays a key role in the development of endothelial dysfunction [3, 17, 27]. Nowadays western diets are high in calories, saturated fat, and simple sugars and are also characterized by an imbalance in the dietary intake of two essential nutrients, n-3 and n-6 PUFA [9, 28]. In this study, we demonstrated for the first time that n-3 PUFA depletion for 12 wks could accelerate the process of endothelium dysfunction in young mice (21-wk-old) in atherosclerosis-prone apoE⁻/⁻ genotype. n-3 PUFA depletion was able to induce endothelial dysfunction, independently of caloric intake and development of obesity. Our data were performed in animals receiving vegetable oil as n-3 PUFA source. Therefore, our conclusion on the role of
of n-3 PUFA depletion only refers to alpha linolenic acid depletion, and not to very long-chain fatty acid depletion, which are both crucial for human health and nutrition.

The n-3 PUFA depletion totally alleviated the orectic action due to the genetically deficient in apoE. Indeed, we showed that apoE/−/− mice presented an elevated food intake compared to WT mice. The dysregulation of food intake can be explained by the fact that, in addition to its hepatic synthesis, apoE is abundantly synthesized in the brain where it can participate to the energy homeostasis [23, 29, 30]. ApoE exerts anorectic function mediated by the central melanocortin system and genetically obese mice or diet-induced obese rats are deficient in brain gene expression of apoE [23, 29].

Adiposity was altered in apoE/−/− versus WT animals. Indeed, apoE/−/− mice fed a chow diet are leaner than WT mice and the fat mass was lower in apoE/−/− mice compared to WT mice. Several other studies have also described similar effects of apoE deficiency on body composition, most of them suggesting an implication of triglyceride reuptake mediated by peripheral apoE synthesis [31–33]. More specifically, the absence of adipose-derived apoE may play a role in intracellular lipid storage, contributing to the modifications of lean versus fat mass observed in apoE/−/− mice [31]. After 12 wks of n-3 PUFA depletion total body weight gain was similar between genotypes and again this alleviated the effect on adiposity observed in apoE-deficient mice. Interestingly, body weight was similar in all conditions at the end of the treatment. apoE/−/− mice are known to be more resistant to obesity than their WT littermates [34]. However, when fed a high-fat diet (critical for the observation of an early impairment of endothelium-dependent relaxation in young adults [17, 35]), they tend to develop overweight or even obesity [36, 37]. In our model, the endothelial dysfunction was observed independently of alterations of body mass.

In addition to the effects on body composition, apoE deficiency promoted the development of hepatic steatosis in response to n-3 PUFA depletion without change in liver tissue weight. The presence of hepatic steatosis has previously been reported for apoE/−/− mice fed a high-fat diet and the model has been proposed to study the features of NAFLD on cardiovascular events [38, 39]. In this study, we confirm the development of hepatic steatosis in WT DEF mice, as described by Pachikian et al. [9]. It is likely that the upregulation of genes involved in lipogenesis, namely FAS and SCD-1 or in CHOL homeostasis, namely Hmgcr and LDLr, accounted for lipid accumulation in the liver of n-3 PUFA-depleted mice. These genes are under the control of two key transcription factors, SREBP-1c and SREBP-2, respectively. Both factors were activated in nuclear fraction in the liver of n-3 PUFA-depleted mice versus mice treated with a CT diet, although it is less pronounced in the liver of apoE-deficient mice.

Neither resting parameters (basal tone and vessel diameter) nor contraction profile (response to KCl challenge or α-adrenoceptor stimulation) of mesenteric arteries were affected by the genetic background or the n-3 PUFA depletion. However, n-3 PUFA depletion for 12 wks was sufficient to alter the endothelial function in apoE/−/− mice, showing an acceleration of vascular alterations, since twice as much time is required to observe such alterations in apoE/−/− fed a chow diet [16, 17]. Our results indicated that prostanooids did not play an important role in this early development of endothelial dysfunction because the presence of indo, a nonselective cyclooxygenase inhibitor, did not induce any effect. As previously reported, impairment of endothelial function mainly relies on a reduced NO bioavailability in apoE/−/− mice models [14, 17]. This is corroborated in n-3 PUFA-depleted apoE-deficient mice as the NOS inhibitor, L-NAME, blunted the vasorelaxation of mesenteric arteries from all mice and abrogated the differences between genotypes, treated and untreated conditions. More, n-3 PUFA depletion in apoE/−/− mice lead to a reduced circulating level of HB-NO compared to their CT mice. Despite the absence of measurable alterations in vascular function, WT DEF mice presented also reduced HB-NO levels compared to their CT mice. It has been previously reported in different experimental models that the development of endothelial dysfunction build slower in WT mice than in apoE genetically deficient mice (despite high-fat diet, body weight gain, and/or development of type 2 diabetes [16, 40, 41]).

As expected, mice deficient in apoE have altered lipid profiles characterized by hypercholesterolemia due to the lack of CHOL reuptake. The depletion in n-3 PUFA therefore resulted in much higher levels of CHOL. Both cardiovascular risks predictors, non-HDLc and HDLc/total CHOL, were increased in apoE/−/− mice compared to WT mice. Once again the dietary depletion in n-3 PUFA in apoE/−/− mice

<table>
<thead>
<tr>
<th>Plasma lipids</th>
<th>WT CT</th>
<th>WT DEF</th>
<th>KO CT</th>
<th>KO DEF</th>
</tr>
</thead>
<tbody>
<tr>
<td>Triglyceridemia (mM)</td>
<td>0.75 ± 0.08</td>
<td>0.52 ± 0.04</td>
<td>0.66 ± 0.08</td>
<td>0.77 ± 0.12</td>
</tr>
<tr>
<td>Total cholesterol (mM)</td>
<td>2.16 ± 0.10</td>
<td>2.37 ± 0.15</td>
<td>4.33 ± 0.92</td>
<td>5.65 ± 0.93**,**$$</td>
</tr>
<tr>
<td>HDL-CHOL (mM)</td>
<td>1.32 ± 0.07</td>
<td>0.87 ± 0.19</td>
<td>0.65 ± 0.13**</td>
<td>0.73 ± 0.10*</td>
</tr>
<tr>
<td>CHOL/HDLc</td>
<td>1.69 ± 0.16</td>
<td>3.02 ± 1.1</td>
<td>6.68 ± 0.42**,**$$</td>
<td>7.45 ± 0.27**,**$$</td>
</tr>
<tr>
<td>Non-HDLc (mM)</td>
<td>0.83 ± 0.16</td>
<td>1.71 ± 0.27</td>
<td>3.45 ± 0.78*</td>
<td>4.9 ± 0.82**,**$$</td>
</tr>
</tbody>
</table>

WT mice fed a CT diet (WT CT, n = 8). WT mice fed an n-3 DEF diet (WT DEF, n = 7). apoE/−/− mice fed a CT diet (KO CT, n = 7) and apoE/−/− mice fed an n-3 DEF diet (KO DEF, n = 7) for 12 wks. Data are expressed as the mean ± SEM. Data were analyzed by one-way ANOVA followed by multiple comparison tests: * versus WT CT, ** versus WT DEF.
worsened these parameters, factors that could also contribute to increased cardiovascular risks, in addition to the alteration of endothelial function. As observed in our previous study [42], WT DEF mice did not display hyperlipidemia compared to their CT mice but both cardiovascular risks predictors were almost doubled compared to their CT mice.

Endothelium dysfunction and atherosclerosis progression in apoE-deficient mice is usually related to chronic inflammation phenomenon [43–45]. In addition, high fat fed apoE−/− mice display an increased expression of circulatory inflammatory factors compared to chow fed mice. In comparison to other studies aiming to evaluating the influence of diet on vascular parameters, we did not observe any induction of inflammatory cytokines in any genotypes. Again, because obesity and hyperlipidemia may both contribute to inflammation, the absence of obesity probably contributed to this less severe inflammatory status [46].

In conclusion, we are able to propose a new experimental murine model to study the early endothelial dysfunction occurring independently of obesity and inflammation and associated with hepatic steatosis. Our model presents several advantages compared to high-fat diet fed apoE−/− mice that make it particularly suitable to investigate critical features of NAFLD: (i) it recapitulates the lipid profile, hepatic phenotype, and endothelial dysfunction; and (ii) it eliminates the confounding parameter of overweight/obesity and inflammation.


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5 References

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