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## **Digestive n-6 Lipid Oxidation, a Key Trigger of Vascular Dysfunction and Atherosclerosis in the Western Diet: Protective Effects of Apple Polyphenols**

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1 **Digestive n-6 lipid oxidation, a key trigger of vascular dysfunction and atherosclerosis in**  
2 **the Western diet: protective effects of apple polyphenols**

3  
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29  
30 **Abbreviations:** 4-HNE: 4-hydroxy-2-nonenal; Ach: Acetylcholine; ApoE: ApolipoproteinE;  
31 EPR: Electron Paramagnetic Resonance; HF: High Fat; NO: Nitric Oxide; oxLDL: oxidized  
32 LDL; PE: Phenolic Extract; PC: Proanthocyanidins; PUFA: polyunsaturated fatty acids; ROS:  
33 Reactive Oxygen Species; RM: Red Meat; SNP: Sodium Nitroprusside; SO: Sunflower Oil;  
34 WD: Western Diet.

35 **Keywords:** 4-Hydroxy-2-nonenal, Atherosclerosis, Endothelial function, Polyphenols,  
36 Western diet

37      **Abstract**

38      **Scope**

39      A main risk factor of atherosclerosis is a Western diet (WD) rich in n-6 PUFA sensitive to  
40      oxidation. Their oxidation can be initiated by heme iron of red meat leading to the formation of  
41      4-hydroxy-2-nonenal (4-HNE), a cytotoxic aldehyde. An increased 4-HNE production is  
42      implicated in endothelial dysfunction and atherosclerosis. By contrast, a diet rich in  
43      proanthocyanidins reduces oxidative stress and arterial diseases. This study evaluates the  
44      effects of a WD on vascular integrity in ApoE<sup>-/-</sup> mice and the protective capacity of apple  
45      extract and puree rich in antioxidant proanthocyanidins.

46      **Methods and results**

47      ApoE<sup>-/-</sup> mice were fed during 12 weeks with a WD with or without n-6 PUFA. Moreover,  
48      two WD + n-6 PUFA groups were supplemented with apple puree or phenolic extract. We  
49      report an increase in digestive 4-HNE production associated with a rise in plasmatic 4-HNE  
50      and oxLDL concentrations. Oxidizable n-6 PUFA consumption was associated with a worsened  
51      endothelial dysfunction and atherosclerosis. Interestingly, supplementations with apple  
52      polyphenol extract or puree prevented these impairments while reducing oxidative stress.

53      **Conclusion**

54      n-6 lipid oxidation during digestion may be a key factor of vascular impairments.  
55      Nevertheless, an antioxidant strategy could limit 4-HNE formation during digestion and thus  
56      durably protect vascular function.

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## 63 INTRODUCTION

64

65 Atherosclerosis, a leading cause of mortality, is a multifactorial and degenerative disease  
66 characterized by the progressive accumulation of lipids and inflammatory cells on the wall of  
67 large and medium arteries. Such changes result in blood flow impairments, chronic  
68 inflammation and oxidative stress in the vascular wall. <sup>[1,2]</sup> Lipid accumulation within the  
69 arterial wall is preceded by an altered vascular endothelial function, which is explained by a  
70 complex interplay between reduced nitric oxide (NO) bioavailability, oxidative stress and  
71 inflammation. <sup>[2,3]</sup> One of the major risk factors of atherosclerosis development is the Western  
72 diet rich in n-6 polyunsaturated fatty acids (n-6 PUFA) which are highly sensitive to oxidation.  
73 In the digestive tract, food is exposed to postprandial oxidative stress and therefore the gastric  
74 tract has been proposed as a major site for lipid oxidation. <sup>[4-6]</sup> Indeed, in the Western diet,  
75 heme iron in the form of myoglobin from red meat can initiate lipid oxidation leading to the  
76 formation of 4-hydroxy-2-nonenal (4-HNE). 4-HNE is a specific genotoxic and cytotoxic  $\alpha,\beta$ -  
77 unsaturated hydroxyalkenal generated from n-6 PUFA and found in some oxidative stress-  
78 related and inflammatory diseases. <sup>[7]</sup> Keller et al. (2015) <sup>[8]</sup> have shown that marked 4-HNE  
79 administered to rats by intragastric gavage was largely absorbed by the intestinal barrier since  
80 it was found in urine, liver, or kidney. <sup>[8]</sup> This highly reactive aldehyde binds covalently (via  
81 Michael addition or Schiff base formation) with free amino or thiol groups of cysteine, lysine  
82 and histidine. <sup>[9]</sup> This adduction results in an impairment of the protein conformation and  
83 function inducing subsequent cellular dysfunctions and tissular damages. As a matter of fact,  
84 the covalent binding of 4-HNE to ApoB100, which yields oxidized LDL (oxLDL), is  
85 recognized as a key step in the development of an endothelial dysfunction and the subsequent  
86 atheromatous plaque formation. <sup>[10,11]</sup>

87 In the last decades, epidemiological, clinical and experimental studies have demonstrated  
88 that a healthy diet plays a central role in the prevention of atherosclerosis. As a matter of fact,  
89 inverse associations are repeatedly outlined between coronary artery disease and stroke and the  
90 consumption of fruit and vegetables. <sup>[12,13]</sup> In recent controlled trials with cocoa and other  
91 flavonoid-rich fruit and vegetables, the vascular function was improved along with the plasma  
92 NO status and inflammation-related markers. <sup>[14,15]</sup> As a matter of fact, proanthocyanidins  
93 (PCs) appear as potential mediators in reducing blood pressure and improving endothelium-  
94 mediated dilation whereas their plasma metabolites were found to reduce monocyte adhesion  
95 to endothelial cells through modulation of gene expression and TNF- $\alpha$ . <sup>[16,17,18,19]</sup> PCs are the  
96 second polyphenol class in the French diet with apple being the major contributor. <sup>[20]</sup>

97 Monomeric and oligomeric PCs represent 80% of the apple phenolic pool followed by  
98 hydroxycinnamic acids (15%), flavonols and dihydrochalcones. After ingestion, native forms  
99 of polyphenols are bioaccessible in the stomach after their release from the plant matrix. [21]  
100 Antioxidant apple polyphenols were found to inhibit lipid oxidation in *in vitro* gastric  
101 digestion. [22] Through their catechol core, they can reduce the pro-oxidant hypervalent iron  
102 form of myoglobin (MbFe<sup>IV</sup>=O) to metmyoglobin (MbFe<sup>III</sup>) as well as chelate free iron  
103 preventing lipid oxidation initiation. [23] Antioxidant effects of apple polyphenols in the  
104 gastrointestinal tract could limit 4-HNE formation and its subsequent absorption. Limiting 4-  
105 HNE absorption can reduce LDL oxidation and thus protect the vascular function.

106

107 The aims of this study were to evaluate 1) the effects of a chronic Western diet and the  
108 subsequent lipid oxidation occurring in the digestive tract on both the vascular function and  
109 atherosclerosis development in ApoE<sup>-/-</sup> mice, and 2) the consequences of a primary prevention  
110 strategy by a supplementation in polyphenols rich in PCs.

111

## 112 MATERIALS AND METHODS

### 113 Animal model and study design

114 All investigations are conformed to the *Guide for the Care and Use of Laboratory Animals*  
115 published by the US National Institutes of Health (NIH, National Academies Press US, 8<sup>th</sup>  
116 edition, 2011) as well as European Parliament Directive 2010/63/EU (experimentation n°:  
117 84.004. Eight weeks old male C57Bl6J mice (n = 100) knockout for apolipoproteinE (ApoE<sup>-/-</sup>)  
118 (Charles River, France / Breeding in HP2 laboratory, Grenoble) were used for this study. Mice  
119 were housed five by cage under controlled conditions of temperature (21 ± 1 °C), hygrometry  
120 (60 ± 10%) and lightening (12:12 hours light-dark cycle), with access to water and food *ad*  
121 *libitum*. After two weeks of acclimatization, male mice were randomly assigned to five groups  
122 (n = 20 per group): a first group with a normocholesterolemic standard diet (SD group) (SAFE  
123 A04), a Western diet group with High Fat with red meat (WD group), a second Western diet  
124 group complemented with sunflower oil (WD+SO group) and two other groups with a  
125 WD+SO diet supplemented with apple polyphenols in the form of apple puree  
126 (WD+SO+PUREE group) or the corresponding phenolic extract (WD+SO+PE group). SD

127 group was only conducted in order to confirm the effects of our Western Diet in a model of  
128 ApoE<sup>-/-</sup> mice. Consequently, differences observed between SD and WD groups are reported in  
129 supplemental data.

130 Diet protocols were sustained during 12 weeks and food intake was quantified as a mean per  
131 cage (n = 3 cages with 6/7 mice per cage) each three days during the whole protocol. During  
132 this period, weight gain was measured every week and dietary intake was evaluated three times  
133 per week. Faeces were harvested at the start, middle and end of the protocol. During the last  
134 week, blood pressure was evaluated by tail cuff method in 12 animals per group and  
135 atheromatous plaque size by echography on the 8 other animals of the group. At the end of the  
136 12 weeks-diet period, an overnight fasting was performed and mice were euthanized by sodium  
137 pentobarbital injection (120 mg/kg I.P.). Blood was collected for biochemical analyses. Hearts  
138 were excised with aortic arch and fixed in O.C.T then frozen in liquid nitrogen and stored at -  
139 80 °C for histological analysis. Finally, thoracic aorta was removed in order to perform *ex-vivo*  
140 analysis of vascular function (n = 8/grp), quantification of atheromatous plaque development  
141 (n = 8 for aortic staining with Oil-Red O) or biochemical assays (n = 4 per group)

142

### 143 **Dietary protocol**

144 Regarding the diet protocol, the first group was fed with a High Fat and red meat diet (HF  
145 230, Safe, France) (Western diet, WD). Composition in % kcal: proteins 16.1, carbohydrates  
146 24.2, lipids 59.7 for a total of 12.1 kcal per day. Composition in % of diet weight: High Fat  
147 79.5, red meat 19.9, cellulose 0.64, n-6 PUFA 10.5, cholesterol 0.076, Vitamin E 0.014 and  
148 heme iron 0.0013. The second group was fed with a WD diet complemented with sunflower oil  
149 (SO, Rustica from Leclerc, lot A21815) as a source of oxidizable n-6 PUFA (WD+SO group).  
150 Composition in % kcal: proteins 12.3, carbohydrates 18.5, lipids 69.2 for a total of 13.0 kcal  
151 per day. Composition in % of diet weight: High Fat 68.6, sunflower oil 13.7, red meat 17.1,  
152 cellulose 0.55, n-6 PUFA 28.3, cholesterol 0.066, Vitamin E 0.021 and heme iron 0.0011. The  
153 2 last groups were fed with a WD+SO diet supplemented with apple (*Reinette de Flandre*,  
154 Ambricourt, France) polyphenols in the form of apple puree (whole apple matrix except seeds)  
155 for the WD+SO+PUREE group or the corresponding phenolic extract for the WD+SO+PE  
156 group. Composition for WD+SO+PUREE group in % kcal: proteins 12.1, carbohydrates 19.4,  
157 lipids 68.5 for a total of 11.7 kcal per day; in % of diet weight: High Fat 60.6, sunflower oil

158 12.1, red meat 15.1, Puree 12.1 (polyphenols 0.060), n-6 PUFA 28.3, cholesterol 0.058,  
159 Vitamin E 0.019 and heme iron 0.0010. Composition for WD+SO+PE group in % kcal:  
160 proteins 12.1, carbohydrates 19.6, lipids 68.3 for a total of 12.4 kcal per day; in % of diet  
161 weight: High Fat 67.0, sunflower oil 13.4, red meat 16.7, extract 2.3 (polyphenols 0.061),  
162 cellulose 0.54, n-6 PUFA 28.3, cholesterol 0.064, Vitamin E 0.021 and heme iron 0.0011.

163

#### 164 **Blood pressure measurements**

165 Before the first week and during the last week of the dietary protocol, systolic, diastolic and  
166 mean blood pressures were assessed in conscious mice by tail-cuff method using CODA tail  
167 cuff system (Kent Scientific, Torrington, CT, USA). To reduce stress influence, mice  
168 underwent 3 periods of habituation to blood pressure measurements during the week preceding  
169 final recording. Mice were warmed for 30 min before measurements so that their skin  
170 temperature reached 33-34 °C during measurements. All measurements were made by the  
171 same experimenter between 9 AM and 12 AM. Obtained results were the mean of at least 10  
172 valid measurements out of the 20 performed in a recording session.

173

#### 174 **Ultrasound imaging (echography) of atheromatous plaque**

175 Echographic assessment was performed using a Vevo 3100™ imaging system (Fujifilm  
176 VisualSonics, Toronto, Canada) equipped with the MX550D™ probe (22-55 MHz). Images  
177 were analyzed post-processing using the VevoLab™ software (Fujifilm VisualSonics, Toronto,  
178 Canada). Echographic assessment as well as image analysis were performed in a blind manner.  
179 Mice anesthesia was induced with 4% isoflurane (IsoFlo™, Zoetis, Parsippany, USA) in  
180 medical air mix (22% O<sub>2</sub>, 78% N<sub>2</sub>) (Air Liquide, Paris, France), ventilated at 1 L/min, and then  
181 maintained with 1.5% isoflurane after effective sleep. Animals were placed in a supine position  
182 on a heating pad and their body temperature was monitored throughout the exam, as well as  
183 their breathing rate and electrocardiogram. Cross-sectional images of ascending aorta were  
184 obtained from a modified right parasternal view. Aortic plaque surface areas were determined  
185 from 3 measurements at the same spot. All measurements were performed between two  
186 inspiratory peaks.

187

188 **Isolated aortic rings and vasoreactivity**

189 Under anesthesia (120 mg/kg I.P.), thoracic aortas were quickly removed and placed in cold  
190 Krebs-Henseleit bicarbonate buffer (composition in mM: NaCl 118, NaHCO<sub>3</sub> 25, KCl 4.8,  
191 KH<sub>2</sub>PO<sub>4</sub> 1.2, CaCl<sub>2</sub> 2.5 and glucose 11). After removal of adherent perivascular adipose  
192 tissues, aortas were cut into 2 mm long rings. Then, aortic rings were set onto stainless steel  
193 connected to an isometric force transducer, linked to an amplifier and a computerized  
194 acquisition system, to record changes in isometric force (EMKA technologies, EMKA Paris,  
195 France). They were suspended in organ bathes containing Krebs-Henseleit buffer at 37 °C  
196 continuously bubbled with gas mixture (95% O<sub>2</sub> – 5% CO<sub>2</sub>). Resting tension was adjusted to  
197 0.8 g and aortic rings were allowed to stabilize during 60 min. During this period, Krebs-  
198 Henseleit buffer was changed two times and rings were re-stretched to maintain a tension of  
199 0.8 g. First, smooth muscle cells and endothelial integrity were assessed by a single dose of  
200 phenylephrine (PE, 10<sup>-6</sup> M) followed by a single vasorelaxing dose of acetylcholine (ACh, 10<sup>-5</sup>  
201 M). Then, each vessel ring was pre-constricted with phenylephrine (10<sup>-6</sup> M). After pre-  
202 constriction reached a plateau, endothelium-dependent and independent relaxations were  
203 examined by challenging aortic rings with cumulative concentrations of ACh (10<sup>-9</sup> to 10<sup>-5</sup> M)  
204 or sodium nitroprusside (SNP, 10<sup>-9</sup> M to 10<sup>-5</sup> M) respectively. Vasodilatation was expressed as  
205 a percentage of maximum contractile response to PE. Maximal relaxation (E<sub>max</sub>) and  
206 sensitivity (EC<sub>50</sub>) to each drug were used to characterize vasorelaxation.

207

208 **Blood analysis**

209 Blood analyses were performed on plasma obtained by centrifuging blood at 300 g for 10  
210 min at 4 °C and plasma was stored at -80 °C for biochemical analyses. Total cholesterol (TC),  
211 high-density lipoprotein cholesterol (HDL) and low-density lipoprotein cholesterol (LDL)  
212 concentrations were determined by the CHOD-PAP method (Biolabo SAS, France).  
213 Triglycerides (TG) were assessed by the GPO method (Biolabo SAS, France). Finally,  
214 malondialdehyde-modified oxLDL were assessed by an ELISA kit (Cloud-Clone Corp., USA).

215

216 **Biochemical assays**

217 *Measurement of reactive oxygen species (ROS) by electron paramagnetic resonance (EPR)*



218 ROS production was measured by EPR in aortic homogenates as previously described. [24]  
219 Briefly, 50  $\mu\text{L}$  of homogenates were incubated with 5  $\mu\text{L}$  of Krebs-Hepes buffer (pH 7.4)  
220 containing 1 mM of CMH (1-hydroxy-3-methoxycarbonyl-2,2,5,5-tetramethylpyrrolidine) and  
221 25  $\mu\text{M}$  deferoxamine methane sulfonate. This solution was placed inside the e-scan  
222 spectrometer (Bruker, Ettlingen, Germany) for data acquisition at 37 °C. The acquisition EPR  
223 parameters were: microwave frequency = 9.652 GHz; modulation frequency = 86 kHz,  
224 modulation amplitude = 0.01 G; center field = 3495.9 G, sweep width = 300 G; microwave  
225 power = 1.0 mW; number of scans = 10 and receiver gain =  $2.10^{-1}$ . Adducts generated by the  
226 probe reaction with free radicals were acquired and the spectra sequentially recorded for about  
227 5 min in order to calculate the ROS production rate. The ROS production was normalized to  
228 the protein content of each sample and then expressed in  $\mu\text{mol min}^{-1}\text{mg}^{-1}$ .

#### 229 *Nitrites in aorta*

230 Nitrites from descending aorta, used as an index of total NO production, were quantified by  
231 using the high-sensitivity nitrite assay kit (Measure-iT™ High-Sensitivity Nitrite Assay Kit;  
232 Invitrogen™) and was performed according to the manufacturer's instructions.  
233 Measurements were performed at the end of 12 weeks of protocol diet. NO production was  
234 expressed relative to protein content of each sample.

235

#### 236 *Quantification of faeces and plasma 4-HNE*

237 Lipid oxidation during digestion was evaluated by the quantification of 4-HNE in faeces and  
238 plasma. Faeces were collected 24 hours after diet change at the start, middle and end of the 12  
239 weeks protocol. Faeces (200 mg) were crushed with mortar and pestle in liquid nitrogen. The  
240 resulting powder (ca. 30 mg) was vortexed once with 500  $\mu\text{L}$  of acetonitrile containing 50 ng  
241 of 4-HNE-D3 (internal standard) during 5 min. After centrifugation (16 000 g, 5 min, room  
242 temperature), the supernatant was reacted with 100  $\mu\text{L}$  of a solution of 2,4-  
243 dinitrophenylhydrazine (75 mg of 2,4-DNPH added with 0.6 mL of formic acid and 10 mL of  
244 acetonitrile) during 1 hour at room temperature. For plasma, 70  $\mu\text{L}$  were vortexed with 200  $\mu\text{L}$   
245 of acetonitrile and 20  $\mu\text{L}$  of 4-HNE-D3 (10 ng/mL in ACN) during 5 min. After centrifugation  
246 as above, the supernatant is dried under nitrogen and reacted with 50  $\mu\text{L}$  of a solution of 2,4-  
247 DNPH (25 mg of 2,4-DNPH added with 0.2 mL of formic acid and 10 mL of acetonitrile)  
248 during 1 hour at room temperature. Separation and quantification of derivatized 4-HNE were

249 performed by using LC/MS/APCI (EVOQ Elite, Bruker Daltonics, Bremen, Germany) with an  
250 ACQUITY HSS T3 column (50 mm x 2.1 mm; 1.7  $\mu\text{m}$ , Waters, Saint-Quentin-en-Yvelines,  
251 France) at 40 °C. For chromatographic separation, the mobile phase was constituted by a  
252 binary solvent system with water/formic acid (99.95/0.05, v/v, solvent A) and acetonitrile  
253 (solvent B) at the flow rate of 0.4 mL/min. The volume injected was 4  $\mu\text{L}$ . The elution gradient  
254 was as follows: 0-1 min isocratic 50% B; 1-9 min, linear 50-98% B; 9-10 min, isocratic 98%  
255 B; 10-10.1 min, linear 98-50% B and 10.1-11 min, isocratic 50% B. Mass spectrometry  
256 conditions were as follows: ionization by APCI in negative mode, spray current 20  $\mu\text{A}$ ; cone  
257 temperature 300 °C; heated probe temperature 300 °C; cone gas flow 20; probe gas flow 40;  
258 nebulizer gas flow 50. Spectra were recorded in the MRM mode. Derivatized 4-HNE-D3 was  
259 quantified by following the transition from ion at  $m/z$  338 to ion at  $m/z$  167 and derivatized 4-  
260 HNE with transition from  $m/z$  335 to  $m/z$  167. The retention time of both compounds was 3.5  
261 min. For quantification, 5 point-calibrations were injected at constant concentration for 4-  
262 HNE-D3 (0.28  $\mu\text{M}$ ) and between 1.02  $\mu\text{M}$  and 0.064  $\mu\text{M}$  for 4-HNE. The adducts with DNPH  
263 were stable over 6 h with a variation lower than 5%.

264

#### 265 *OIL red coloration*

266 Atheromatous plaque size was assessed by measuring lipid aggregation in thoraco-  
267 abdominal aortas that were fixed in paraformaldehyde (4%) during 24 hours at 4 °C. Then,  
268 aortas were rinsed three times with Krebs-Henseleit buffer and incubated overnight in an Oil-  
269 Red-O solution in isopropanol/water (60/40, v/v, 0.33% of Oil-Red-O, Sigma-Aldrich, Saint-  
270 Louis, USA) at room temperature. Atheromatous plaque size was assessed by capturing images  
271 directly from a color camera (Motic Moticam 2300, Motic China group Co.) attached to a  
272 binocular loupe. Images were displayed on a RGB monitor by using Motic Image 2.0 (Motic  
273 China group Co.). Analysis was carried out using Image J<sup>®</sup> software (Image J, NIH, USA). Oil  
274 red staining was expressed as the percentage of aortic area.

275

#### 276 *Histological sections and colorations*

277 The upper part of the heart (with aortic root) was cut using a cryostat (Leica CM1950; Leica  
278 systems, France) at -20 °C. The distal portion of the aortic sinus was recognized by the  
279 appearance of the three leaflets of the valves. Sections of 8  $\mu\text{m}$  thickness were performed. For

280 each aortic root, we quantified atheromatous plaque size and necrotic core size (acellular area)  
281 with Oil-Red-O and Hematoxylin and Eosin staining (Sigma-Aldrich, France) and fibrosis  
282 (Red Sirius, Labomoderne, Paris, France) from five sections separated from each other by 80  
283  $\mu\text{m}$ . Photography was performed using a digital camera (Baumer VCXU31C, Baumer SAS  
284 France), mounted with a macro lens VS Technology 0513 (VS Technology Corporation,  
285 Japan) and Baumer GAPI acquisition software (Baumer SAS France). Quantitative analyses  
286 were performed blindly using Image J<sup>®</sup> software to assess the stained area in  $\text{mm}^2$  and express  
287 it relative to the diameter of aorta in mm (atheromatous plaque size and necrotic core size) or  
288 in percentage of atheroma (fibrosis).

289

## 290 **Statistical analysis**

291 Data were expressed as mean  $\pm$  SEM. Normality was evaluated using D'Agostino and  
292 Pearson test when  $n \geq 8$ . Otherwise, Shapiro-Wilk test was performed. When applicable,  
293 comparison of multiple experimental conditions were performed using analysis of variance  
294 (ANOVA) or repeated measures ANOVA followed by a Tukey post hoc test. A value of  
295  $p < 0.05$  was considered statistically significant. Statistics were performed using GraphPad  
296 Prism 8 (GraphPad Software, La Jolla, USA).

297

298

## 299 **RESULTS**

### 300 **Diet Characterization**

301 ApoE<sup>-/-</sup> mice, classically used to evaluate atherosclerosis development, were distributed to  
302 four groups: a group with a high fat and red meat diet (WD), a second group with a WD diet  
303 complemented with sunflower oil as a source of oxidizable n-6 PUFA (WD+SO group) and  
304 two other groups with a WD+SO diet supplemented with apple polyphenols in the presence of  
305 the apple matrix (Puree) or as a phenolic extract, respectively WD+SO+PUREE and  
306 WD+SO+PE groups. In parallel, a negative control group of ApoE<sup>-/-</sup> mice fed with standard  
307 diet was performed (SD,  $n=20$ , SAFE A04) to evaluate the deleterious effects of WD  
308 (Supplementary data Figure 2).

309 As previously reported, the composition of the sunflower oil used was: 9.8% of saturated  
310 fatty acids (6.3% C16; 3.3% C18; 0.2% C20), 27.8% of monounsaturated fatty acids (0.1%

311 C16:1; 27.0% C18:1n-9; 0.6% C18:1n-7; 0.1% C20:1) and 62.4% of polyunsaturated fatty  
312 acids (62.4% C18:2; 0.01% C18:3).<sup>[25]</sup> The  $\alpha$ -tocopherol content was 665 ppm.

313 Identification and quantification of apple polyphenols were performed by UPLC/DAD/ESI-  
314 MS and thioacidolysis as previously reported.<sup>[25]</sup> Forty-six compounds distributed in four  
315 phenolic classes were identified: proanthocyanidins, hydroxycinnamic acids, dihydrochalcones  
316 and flavonols. The selected variety (*Reinette de Flandre*) is markedly rich in polyphenols (4.3  
317 g/kg) compared to classically consumed dessert apples (1 g/ kg).<sup>[26]</sup> Polyphenols were even  
318 more concentrated in puree after thermal treatment of *Reinette de Flandre* reaching 5 g/kg.  
319 Monomeric and oligomeric PCs represented 5-7% and 67-68%, respectively, of the phenolic  
320 pool followed by the hydroxycinnamic acid, 5-caffeoylquinic acid (21-22%). Quercetin  
321 derivatives and dihydrochalcones were only minor contributors.<sup>[21]</sup> In our model of apple  
322 supplementation, no major difference was observed for polyphenol contents and composition  
323 between Puree and PE. Apples used to prepare the diet also contained, 3.15 g of glucose, 5.85  
324 g of fructose, 1.09 g of saccharose, 0.04 g of citric acid, 1.61 g of malic acid, 17.7 mg of  
325 dehydroascorbic acid and 5.9 mg of ascorbic acid per 100 g of fresh fruit.

326

### 327 **Lipid oxidation during digestion and blood oxidative state**

328 In our model of Western Diets (high fat associated with red meat), body weight increased  
329 all along the protocol to the same extent in the 4 groups (Figures 1A and 1B). In line with  
330 these results, we observed no difference regarding adiposity index defined as (epididymal fat  
331 + inguinal fat + perinephric fat)/mouse weight (Figure 1C), blood HDL (Figure 1D), LDL  
332 (Figure 1E), HDL-C/LDL-C (Figure 1F), total cholesterol (Figure 1G) and triglycerides  
333 (Figure 1H) between groups. These results suggest that there is no difference between groups  
334 regarding lipid absorption. In addition, no difference between groups in term of fasting  
335 glycemia as well as glucose and insulin tolerance (supplemental data) was observed. To  
336 evaluate the impact of a Western diet enriched in n-6 PUFA on lipid oxidation during  
337 digestion and the ability of Puree and PE to modulate this phenomenon, we next measured the  
338 level of 4-HNE in faeces. Before the beginning of the diet (Week 0), only low contents of 4-  
339 HNE were reported at the detection limit of LC/MS/APCI (Figure 2A). After 6 (Figure 2B)  
340 and 12 (Figure 2C) weeks of diet a stronger increase in 4-HNE was observed in the group  
341 supplemented with SO (WD+SO) compared to the WD group. It is worth noting that 4-HNE  
342 was neither detected in freshly reconstituted diets nor after 48 hours at room temperature (data

343 not shown). This suggests that lipid oxidation can occur during *in vivo* gastrointestinal  
344 digestion. The higher levels of 4-HNE in WD+SO compared to WD can be linked to the diet  
345 contents in n-6 PUFA, which are respectively of 28.3% and 10.5% for the WD+SO and WD  
346 diets. Interestingly, the increase observed by the addition of n-6 PUFA was largely prevented  
347 by both Puree and PE supplementations (Figure 2BC). Next, to evaluate whether lipid  
348 oxidation products were also observed in systemic circulation, we measured, after 12 weeks  
349 of diet, plasmatic concentrations of 4-HNE also known to be reliable biomarkers of oxidative  
350 stress. <sup>[9]</sup> As reported in Figures 2D and E, plasmatic level of 4-HNE was higher in the  
351 WD+SO group compared to WD by 87%. In line with the results obtained on 4-HNE in  
352 faeces, this phenomenon was blunted by both apple Puree and PE supplementations by 32%  
353 and 51%, respectively (Figure 2D). Finally, we measured oxLDL, another biomarker of  
354 oxidative stress, which is also known as a strong biomarker of cardiovascular risk. <sup>[10]</sup> The  
355 same pattern was observed since oxLDL level was significantly increased by 14% in the  
356 WD+SO group compared to WD (Figure 2E) while the supplementation by both Puree and  
357 PE markedly attenuated this phenomenon (-22% and -29%, respectively) (Figure 2E).

358

359

#### 360 **4-HNE and cardiovascular risk factors**

361 High levels of 4-HNE and oxLDL are associated in the literature with an increased  
362 cardiovascular risk. <sup>[10]</sup> Then, we evaluated how our Western diet model enriched in n-6  
363 PUFA, supplemented or not with apple polyphenols, affected some key elements of  
364 cardiovascular risk such as arterial blood pressure, endothelial function and finally  
365 atheromatous plaque development. We first evaluated arterial blood pressure by the tail-cuff  
366 method. When the Western diet was enriched with n-6 PUFA (WD+SO), both systolic and  
367 mean arterial pressures were increased compared to WD (Figure 3AC). However, no change  
368 in arterial diastolic pressure was observed between WD and WD+SO groups (Figure 3B).  
369 When the WD+SO diet was supplemented with either Puree or PE, systolic, diastolic and  
370 mean arterial blood pressures were significantly lower than in WD+SO group (Figure 3ABC).

371 Another key element of increased cardiovascular risk factor, which can contribute to the  
372 regulation of blood arterial pressure is endothelial function. Thus, we next evaluated the  
373 impact of our experimental conditions on vascular endothelial function on isolated aortic  
374 rings. In the aorta of animals fed for 12 weeks with a WD enriched in n-6 PUFA, maximal

375 vascular relaxation in response to ACh was reduced by 16% compared to WD (Figure 3D left  
376 and central panel). Considering that no difference was observed between these 2 groups  
377 regarding the response to SNP (Figure 3D right panel), these results strongly support that a  
378 Western diet, enriched in n-6 PUFA, is associated with a vascular endothelium impairment  
379 without smooth muscle cell alteration. In line with our previous results, endothelial  
380 dysfunction was prevented when the animals were supplemented with both apple Puree and  
381 polyphenol extract (Figure 3D left and central panel). Indeed, the maximal response to ACh  
382 was increased by 41% in WD+SO+PUREE and 40% in WD+SO+PE groups compared to  
383 WD+SO. An interesting point is that contrary to the WD+SO group which presented an  
384 impairment of vasodilation to ACh in 75% of the tested arteries (with an impairment threshold  
385 of < 70% of precontraction), no impaired aorta was reported in both supplemented groups.  
386 No impact on the dose-response to SNP was observed.

387 Since, endothelial dysfunction is a key trigger in the pathogenesis of atherosclerosis <sup>[10]</sup> we  
388 next assessed the consequences of endothelial dysfunction on the development of  
389 atherosclerosis. In our study, all experiments have been performed on ApoE<sup>-/-</sup> mice, which are  
390 prompt to develop subsequent atherosclerotic lesions at aortic bifurcations in the aortic arch,  
391 in the descending aorta and in other large arteries. <sup>[27]</sup> The enrichment of WD with n-6 PUFA  
392 increased atheromatous plaque size in aortic arch section as measured by echography (Figure  
393 4A). However, no difference in atherosclerosis was observed by Oil-Red-O coloration in  
394 descending thoraco-abdominal aorta or by histological staining of aortic root in this WD+SO  
395 group when compared to the WD group (Figure 4BC). To evaluate plaque stability,  
396 percentage of fibrosis and necrotic core area size in atheromatous plaque were assessed. No  
397 difference between any groups was observed regarding fibrosis (Figure 4D). However, we  
398 observed that the necrotic core area increased in mice by 52% compared to the WD group  
399 when the Western diet was enriched in n-6 PUFA (Figure 4E). In line with the protective  
400 effect of both apple Puree and PE on endothelial dysfunction, reduced atheromatous plaque  
401 developments were reported by echography in aortic cross (respectively by -20% and -20%)  
402 and by Oil-Red O histological staining in aortic root (respectively by -73% and -37%) as well  
403 as by Oil-Red O staining in thoraco-abdominal aorta (respectively -51% and -45% when  
404 compared to WD+SO) (Figure 4ABC). Another interesting result was that both apple Puree  
405 and PE were able to prevent the increase of the necrotic core area size by 55% and 42%,  
406 respectively (Figure 4E). This last result indicates that plaque stability, which is affected  
407 negatively in mice fed with WD+SO, was preserved with polyphenol supplementation.

408

409 **ROS production and NO pathway in endothelial function**

410 In the vascular wall, the sensitive equilibrium between NO bioavailability and ROS  
411 production contributes mainly to the regulation of the endothelial function. Moreover,  
412 increased ROS production and alteration in NO bioactivity are known to be the key triggers of  
413 endothelial dysfunction and atherosclerosis. [28] Thus, we measured the impact of our  
414 experimental conditions on NO and ROS production in the aortic wall as well as eNOS  
415 expression and phosphorylation on its main activation site serine<sup>1177</sup> (eNOS<sup>Ser1177</sup>). No  
416 difference was observed between WD and WD+SO groups regarding total eNOS expression  
417 and phosphorylation on Ser<sup>1177</sup> (Suppl Figure 2). However, with both supplementations,  
418 eNOS level tended to be lower when compared to the WD+SO group (p=0.06 and 0.08) and  
419 they both seem to increase eNOS activation state through its phosphorylation on serine<sup>1177</sup>  
420 (Suppl Figure 2). To evaluate the impact of our experimental conditions on NO  
421 bioavailability, we next measured the level of nitrites in aortic tissues. We report an increased  
422 nitrite level in WD+SO group compared to WD mice (Figure 5A). Neither Puree  
423 (WD+SO+PUREE) nor polyphenols supplementations (WD+SO+PE) were able to impact  
424 nitrite levels compared to WD+SO group. Finally, since in pro-oxidative conditions, ROS can  
425 react with NO to generate nitro-oxidative stress, which can also contribute to reduce NO  
426 bioavailability, we also measured ROS production by EPR. We report that WD+SO led to  
427 increased ROS production compared to WD (Figure 5B). By contrast, apple Puree  
428 significantly reduced ROS production by 30% whereas polyphenol extract only tended to  
429 reduce it (-26 %, p=0.08) when compared to the WD+SO diet. No statistical difference  
430 between PUREE and PE groups could be further evidenced. Altogether, these results point out  
431 that despite the beneficial effect of apple polyphenol supplementations on endothelial  
432 function, their impact on the NO pathway appears limited. Nonetheless, the modulation of  
433 aortic ROS production may contribute to explain some changes in NO bioavailability in our  
434 groups.

435

436

437        **DISCUSSION**

438        The aim of this study was to give an insight into the mechanism involved in vascular  
439 dysfunction induced by the consumption of a Western diet enriched with n-6 PUFA. ApoE<sup>-/-</sup>  
440 mouse is the most widely used animal model to evaluate the effects of a diet on  
441 atherosclerosis development. ApoE<sup>-/-</sup> mice display an increased peroxidation of lipoproteins,  
442 an elevated level of endothelial cell adhesion molecules, an increased proliferation and  
443 migration of VSMCs as well as a decreased anti-atherogenic function. [29] Moreover, the anti-  
444 inflammatory properties of APOE, combined with its impact on lipoprotein metabolism,  
445 explain why ApoE knockout mice display a very strong development of atherosclerosis  
446 compared to other mouse models. [30-32] However, this model has imperfections and other  
447 models such as LDLr<sup>-/-</sup> have been considered. Nonetheless, our diets have a low cholesterol  
448 content (<0.01% of diet weight) and it has been reported that long diets with higher  
449 cholesterol contents are needed to induce significant lesion development in LDLr<sup>-/-</sup> mice.  
450 [33,34] Consequently, the ApoE<sup>-/-</sup> model appeared as the most appropriate to study the link  
451 between lipid oxidation, endothelial function and atherosclerosis development with our diets.

452        The main results of our work with ApoE<sup>-/-</sup> mice showed that oxidative stress generated in  
453 the gastrointestinal tract (4-HNE) is correlated with an increased cardiovascular risk  
454 (endothelial dysfunction and atherosclerosis) and that preventing lipid oxidation could avoid  
455 these impairments.

456        Lipid oxidation products can be generated *in vivo* and the gastric tract has been proposed as  
457 the major site for diet-induced lipid oxidation. [35] This was confirmed in our study since we  
458 observed that enrichment of a Western diet with n-6 PUFA strongly increases the formation of  
459 4-HNE during digestion, as shown by the higher recovery of 4-HNE in faeces from WD+SO  
460 group. This result is in line with previous studies reporting that a Western meal based on  
461 sunflower oil and beef led to the formation of TBARS, lipid-derived conjugated dienes and 4-  
462 HNE during gastric digestion in mini-pig. [36,37]

463        In order to prevent the deleterious effects of 4-HNE (oxidative stress, cell proliferation, or  
464 cell death), [38] we considered using an antioxidant strategy. Since, we previously reported that  
465 an apple phenolic extract and an apple matrix (Puree) were able to reduce lipid oxidation and  
466 4-HNE formation in an *in vitro* model of digestion, [25] we tried to use both of them *in vivo*.  
467 By adding either the whole apple matrix (WD+SO+PUREE) or the corresponding phenolic  
468 extract (PE) to the diet, we managed to prevent lipid oxidation during digestion. We can



469 hypothesize that in our model, a reduction of the pro-oxidant hypervalent heme iron form  
470 ( $\text{MbFe}^{\text{IV}}=\text{O}$ ) into metmyoglobin ( $\text{MbFe}^{\text{III}}$ ) by chlorogenic acid as well as by monomeric and  
471 oligomeric PCs may have occurred. This reduction inhibits the initiation phase of the lipid  
472 oxidation process and has been previously reported as a major antioxidant effect of PCs and  
473 hydroxycinnamic acids in *in vitro* digestion. <sup>[5]</sup> A remarkable result is that both  
474 WD+SO+PUREE and WD+SO+PE managed to prevent digestive lipid oxidation with the  
475 same extent. This result could be explained by an increased polyphenol bioaccessibility in  
476 WD+SO+PUREE group when compared to raw fruit that we have reported in a previous  
477 work. <sup>[25]</sup> The relative similarity in polyphenol bioaccessibilities for both apple matrix and  
478 phenolic extract may explain the very similar results obtained throughout this study for both  
479 supplementations.

480 Next, we hypothesized that lipid oxidation products generated during digestion may have  
481 an effect on vascular health (endothelial dysfunction, atherosclerosis) after their absorption.  
482 Indeed, we report here that higher lipid oxidation in the digestive tract (4-HNE) is associated  
483 with a higher plasmatic concentration of 4-HNE. These data are in accordance with results  
484 from Keller et al. who observed 4-HNE in urine and various organs (liver, kidney, brain,  
485 heart) after its ingestion. <sup>[8]</sup> It further indicates that even if the majority of 4-HNE could be  
486 excreted in faeces, a part of it is absorbed and can be implicated in vascular impairments.  
487 Moreover, it has been shown that eating a fatty diet can alter the intestinal barrier  
488 physiological state by increasing its permeability <sup>[39]</sup> which could lead to an increased  
489 absorption of 4-HNE.

490 In our work, fasting plasma concentration of 4-HNE after chronic WD+SO diet was 2.7  
491  $\mu\text{M}$  (0.43 mg/L). Such concentration of 4-HNE is known to trigger a variety of biological  
492 responses such as inflammation and cell proliferation. <sup>[9]</sup> Moreover, this plasmatic level of 4-  
493 HNE is also known to induce oxidative stress in the vascular system. It is important to note  
494 that 4-HNE values observed in our work were assessed after a prolonged fasting state and that  
495 we can expect higher values in the postprandial state. Nevertheless, the increased oxidative  
496 stress was confirmed here since the group with the higher 4-HNE concentration displays an  
497 increase in ROS production and plasmatic malondialdehyde-modified oxLDL. Yet, an  
498 increase in oxLDL production has been largely reported in the literature as a key step in the  
499 development of vascular impairments such as endothelial dysfunction or atherosclerosis,  
500 which are generally described as the consequence of oxidative stress. <sup>[40,41]</sup> Moreover, it is  
501 considered that high NO levels can account for nitro-oxidative stress in obese mice. <sup>[42]</sup> The

502 high levels of nitrites observed in the WD+SO group may be the results of the reaction  
503 between NO and superoxide to form peroxynitrite, a potent cytotoxic reactive species. Such a  
504 nitro-oxidative stress is largely reported as a key factor of vascular endothelial impairments  
505 and atherosclerosis. <sup>[43]</sup> Consequently, it was not surprising to observe the worst endothelial  
506 function and the largest atherosclerosis development in the group with the highest lipid  
507 oxidation.

508 We previously pointed out the deleterious effects of digestive lipid oxidation on vascular  
509 integrity and the fact that both WD+SO+PUREE and WD+SO+PE diets were able to prevent  
510 lipid oxidation. We therefore hypothesized that inhibition of lipid oxidation by polyphenol  
511 consumption may prevent vascular impairments. This hypothesis was confirmed regarding the  
512 decrease of ROS production and vascular impairments (reduced endothelial impairments and  
513 atherosclerosis development). Surprisingly, vascular function was not only restored but even  
514 positively enhanced in both supplemented groups. Few assumptions can be performed to  
515 explain the beneficial effect of our diets on vascular function. In a first place, our protocol  
516 does not allow us to exclude a cell-mediated effect of polyphenols included in the extract and  
517 puree since these supplementation were not tested on a group with a standard diet. Indeed, a  
518 direct effect of phase-II and colonic metabolites from proanthocyanidins on vascular function  
519 could not be excluded according to our results and the literature. <sup>[19,44,45]</sup> Nonetheless, it has  
520 been previously reported that 4-HNE was responsible for an impairment of Akt  
521 phosphorylation, which is a main regulator of vascular function especially through eNOS  
522 phosphorylation on Ser1177. <sup>[46]</sup> Despite eNOS phosphorylation on its activation site seems to  
523 be increased in both WD+SO+PUREE and WD+SO+PE groups, the extent of this increase  
524 was not satisfactory and certainly did not appear to be a key element to explain our results on  
525 endothelial function. This idea is reinforced by the lack of effect of both PUREE and PE on  
526 nitrites level when compared to the WD+SO group. Nevertheless, we have to consider that  
527 even if nitrite levels are unchanged, a decrease of oxidative stress can result in a reduction of  
528 NO reaction with  $O_2^-$  to form  $ONOO^-$ . Thus, considering that both PUREE and PE treatment  
529 reduced or tended to reduce ROS production in the vascular wall, we can hypothesize that the  
530 bioavailability of NO may be increased. This hypothesis is supported by the impact of our  
531 experimental conditions on endothelial vasodilatation to ACh. Indeed, on aortic tissue  
532 endothelium-dependent vasodilatation is almost exclusively dependent of the eNOS-NO  
533 pathway, since the pre-incubation of L-NAME almost completely abolishes it in aortas of

534 ApoE<sup>-/-</sup> mice. <sup>[47-49]</sup> Further studies, evaluating eNOS coupling, NO bioavailability and the  
535 level of nitro-oxidative stress will be needed to confirm this point.

536 These results are in accordance with numerous studies reporting the interest of a chronic  
537 uptake of apple polyphenols to reduce risk factors (arterial pressure, blood lipid profile,  
538 inflammation, oxidative stress) and cardiovascular diseases (anti-angiogenic, anti-atherogenic  
539 and anti-hypertensive) in rodents or humans. <sup>[45,50-53]</sup> However, there is no study as this time  
540 that are able to link these effects with a potential antioxidant effect during digestion.

541

## 542 **CONCLUSION**

543 In conclusion, a Western diet rich in n-6 PUFA appears to exacerbate lipid oxidation  
544 during digestion leading to the formation of 4-HNE among other lipid oxidation products. The  
545 absorption of these products is associated with plasmatic oxidative stress, endothelial  
546 dysfunction and development of atherosclerosis in the ApoE<sup>-/-</sup> mice model. The involvement  
547 of digestive lipid oxidation in these phenomena was confirmed since the inhibition of lipid  
548 oxidation and, consequently, 4-HNE synthesis in the gastrointestinal tract, by a  
549 supplementation in apple polyphenols under two different matrix forms, remarkably prevents  
550 those impairments. Consequently, the consumption of proanthocyanidin-rich fruits such as  
551 apple or pear should be encouraged to attenuate the deleterious effect of the Western diet.

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553

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564

565 **Disclosures**

566 Authors declare that they have no conflict of interest. Claire Dufour and Gregory Meyer  
567 designed the study protocols and secured funding.

568

569 **Author Contributions**

570 G.B.; C.P.; A.H.; M.D.; C.G.; S.R.; A-L.C. B.G. G.M. performed experiments. C.A.  
571 developed ApoE<sup>-/-</sup> model and histological protocols. G.B., G.M., C.D. designed the scope,  
572 analyzed the data and wrote the manuscript. C.R., C.A. and B.G. revised the manuscript. All  
573 authors read and approved the final manuscript.

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672

## Figure Legends

673 **Figure 1.** Effects of a Western diet with or without supplementation on weight gain and plasma  
674 lipoproteins. A: Mean of daily food intake during 12 weeks per mice. B: Follow up of body  
675 mass during the 12 weeks of different diets. C: Adiposity index at the end of the protocol diets.  
676 Plasma HDL (D), LDL (E), HDL-cholesterol/LDL-cholesterol (F), total cholesterol (G) and  
677 triglyceride (H), were assessed at the end of the dietary protocols. Values are expressed as  
678 means  $\pm$  SEM (n=6/8 per group for LDL, HDL, HDL-c/LDL-c, total cholesterol and  
679 triglyceride). One way ANOVA (Tukey's multiple comparisons test).

680

681 **Figure 2.** 4-Hydroxy-2-nonenal (4-HNE) formation during gastrointestinal digestion and lipid  
682 oxidation products in plasma. 4-HNE concentration in faeces before (A), at 6 weeks (B) and 12  
683 weeks (C) of diet measured by LC/MS/APCI. Plasma 4-HNE (D) and oxLDL (E) at the end of  
684 the 12 weeks. Values are expressed as means  $\pm$  SEM (plasma 4-HNE n=5/7 per grp, oxLDL  
685 n=6/7 per grp). \*P<0.05 vs. WD. #P<0.05 vs. WD+SO. §P<0.05 vs. WD. \$P<0.05 vs.  
686 WD+SO+PUREE. One way ANOVA (Tukey's multiple comparisons test).

687

688 **Figure 3.** Effects of Western diets and protection by apple polyphenol supplementation on  
689 vascular function. Systolic (A), diastolic (B) and mean (C) blood pressure measured at the end  
690 of dietary protocols by tail cuff method. D: Dose-dependent response to cumulative doses of  
691 acetylcholine (ACh) and sodium nitroprusside (SNP) on aortic rings. *Left:* Dose-dependent  
692 response to cumulative doses of ACh. *Middle:* Maximal relaxation of aortic rings to ACh.  
693 *Right:* Dose-dependent response to cumulative doses of sodium nitroprusside (SNP). Data are  
694 expressed in percent of relaxation relative to maximal contraction obtained with 1  $\mu$ M of  
695 phenylephrine. Values are expressed as means  $\pm$  SEM (blood pressure n=9/11 per group,  
696 vasoreactivity n=6/8 per group). \*P<0.05 vs. WD group. #P<0.05 vs. WD+SO group. §P<0.05  
697 vs. WD. \$P<0.05 vs. WD+SO+PUREE. £P<0.05 vs. Week 0. Dose-response were analyzed  
698 with a repeated measures ANOVA followed with a Tukey multiple comparisons test. Blood  
699 pressures and maximal relaxation were analyzed using a one way ANOVA (Tukey's multiple  
700 comparisons test.)

701

702 **Figure 4.** Development and characterization of atheromatous plaque size induced by Western  
703 diets and protection by apple polyphenol supplementation. (A) Evaluation of aortic cross  
704 atheromatous plaque size performed by echography. (B) Atheromatous plaque size in  
705 descending thoraco-abdominal aorta assessed by Oil-Red-O coloration. (C) Atheromatous  
706 plaque size in aortic root relative to aortic diameter. (D) Percent of fibrosis in atheromatous  
707 plaque of aortic valve and (E) Necrotic core size in atheromatous plaque of aortic root relative  
708 to aortic diameter assessed on histological sections. All measurements were performed at the  
709 end of dietary protocol. Values are expressed as means  $\pm$  SEM (echography n=8 per group,  
710 histological sections n=5 animals per group). One way ANOVA (Tukey's multiple  
711 comparisons test.) \*P<0.05 vs. WD group. #P<0.05 vs. WD+SO group. §P<0.05 vs. WD.  
712 \$P<0.05 vs. WD+SO+PUREE.

713

714 **Figure 5.** Dietary effects on vascular NO and ROS production. (A) Quantification of nitrite in  
715 aorta. (B) Total ROS production measured by electron paramagnetic resonance in aortic tissue

716 homogenate. Values are expressed as means  $\pm$  SEM (nitrite and ROS production n=4/grp).  
717 \*P<0.05 vs. WD group. #P<0.05 vs. WD+SO group. §P<0.05 vs. WD. \$P<0.05 vs.  
718 WD+SO+PUREE. ROS and Nitrite productions were analyzed using a one way ANOVA  
719 (Tukey's multiple comparisons test.)

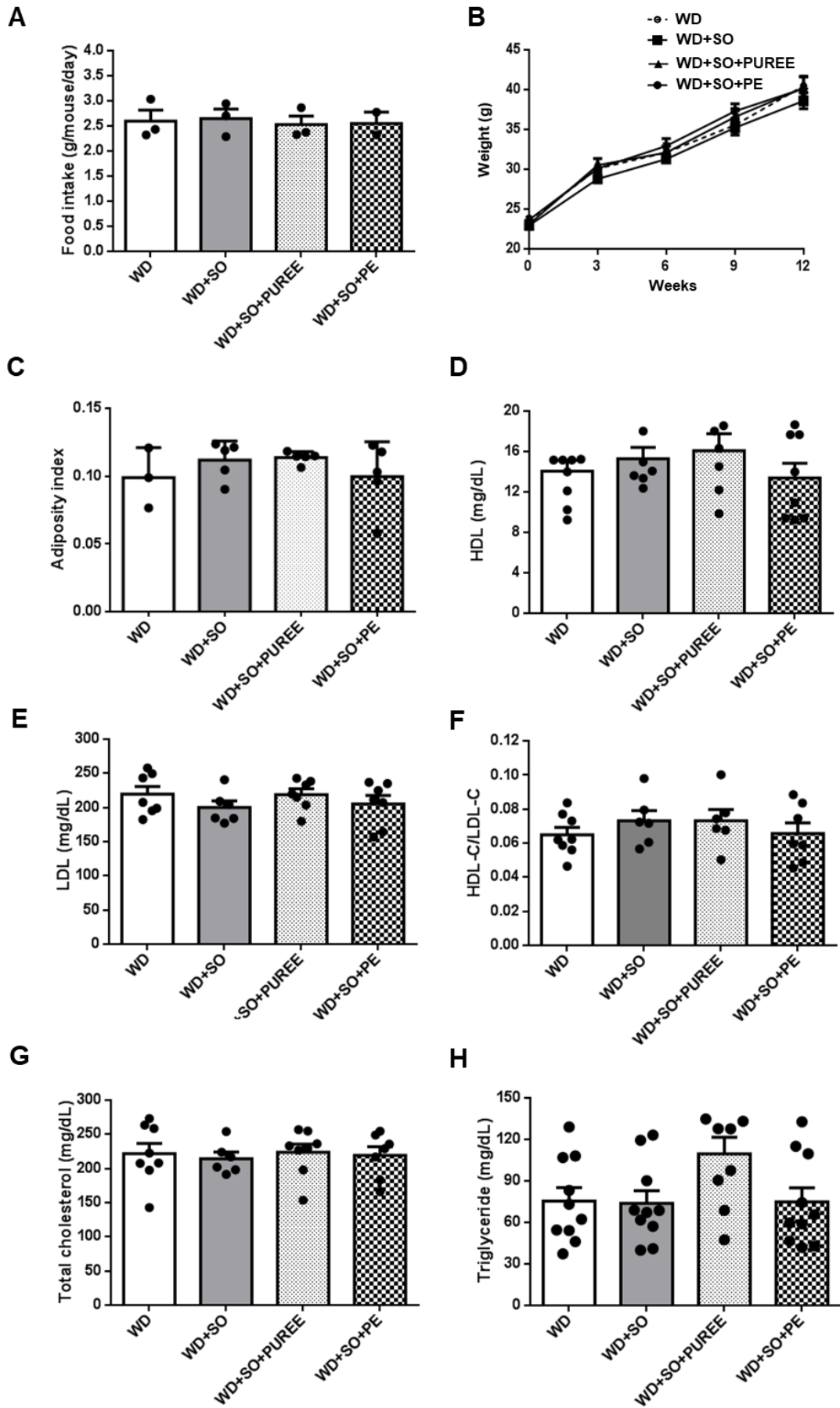
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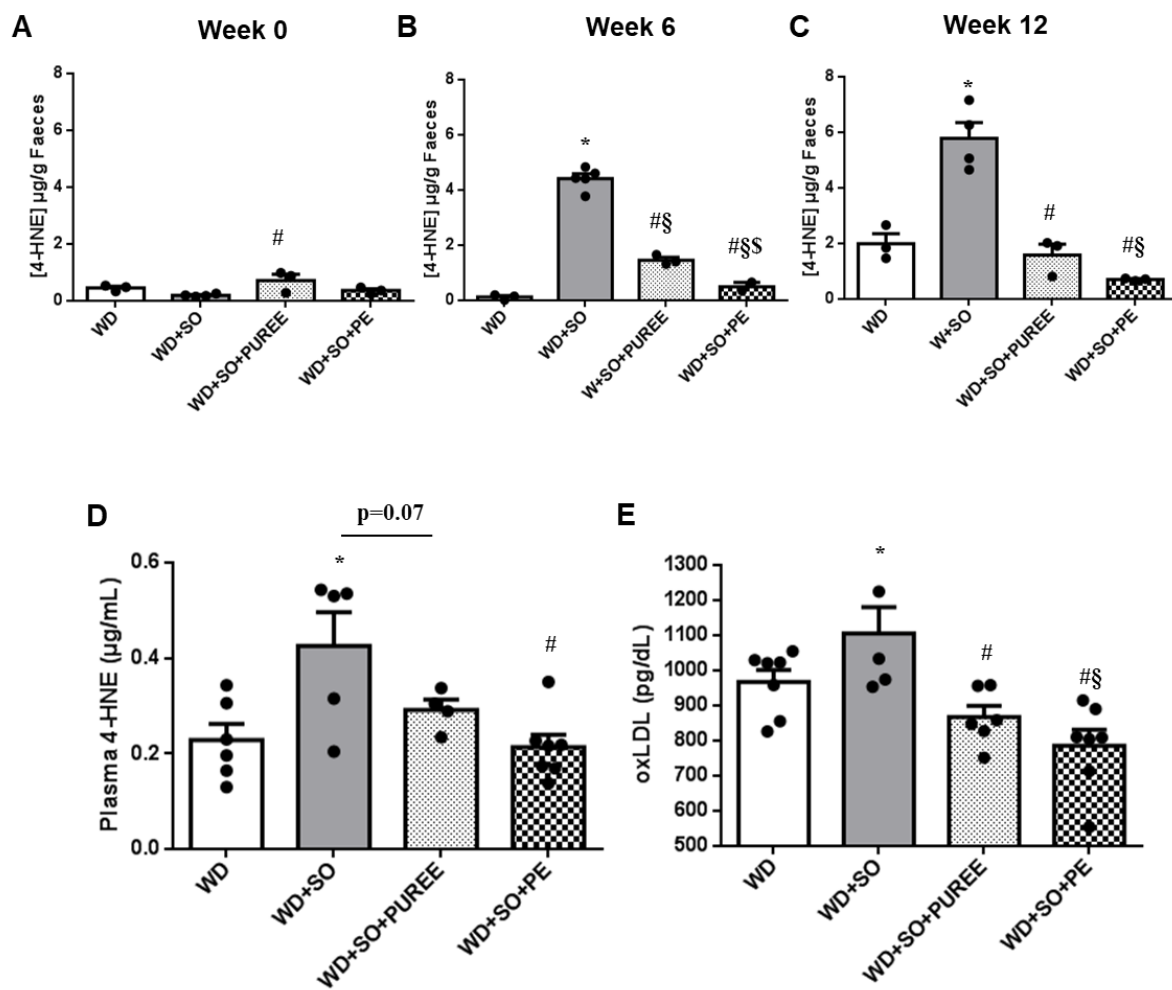
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FIGURE 1



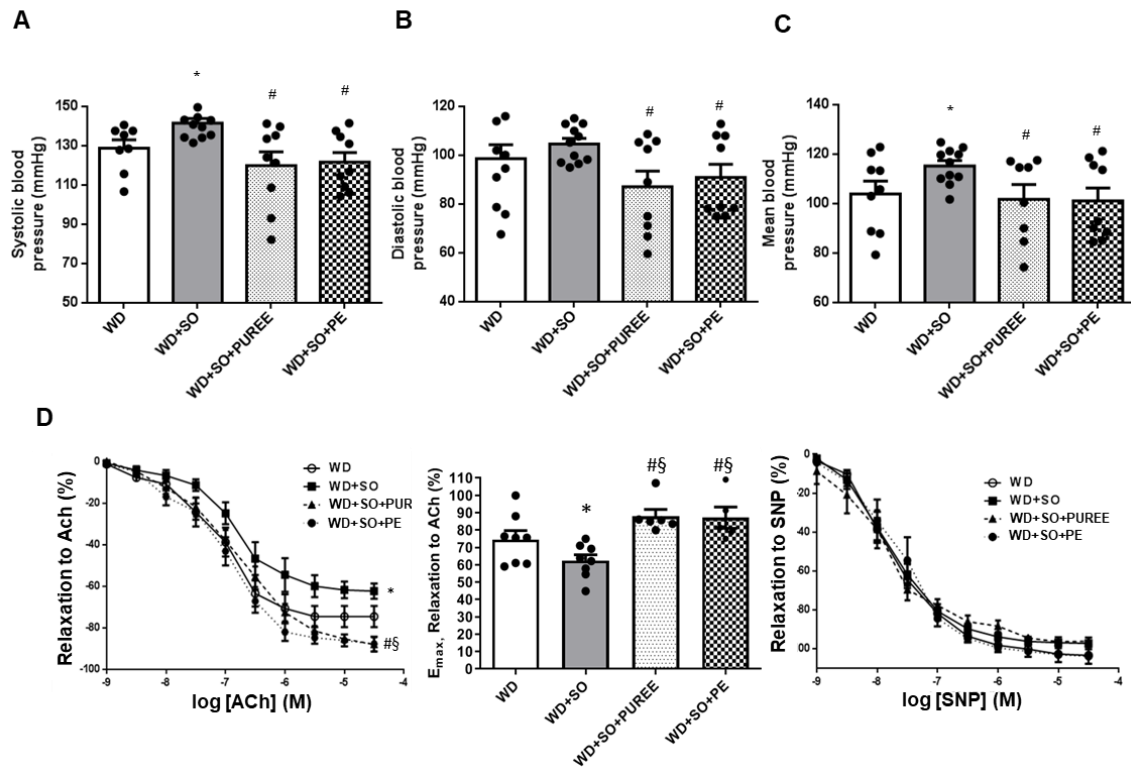
**FIGURE 2**



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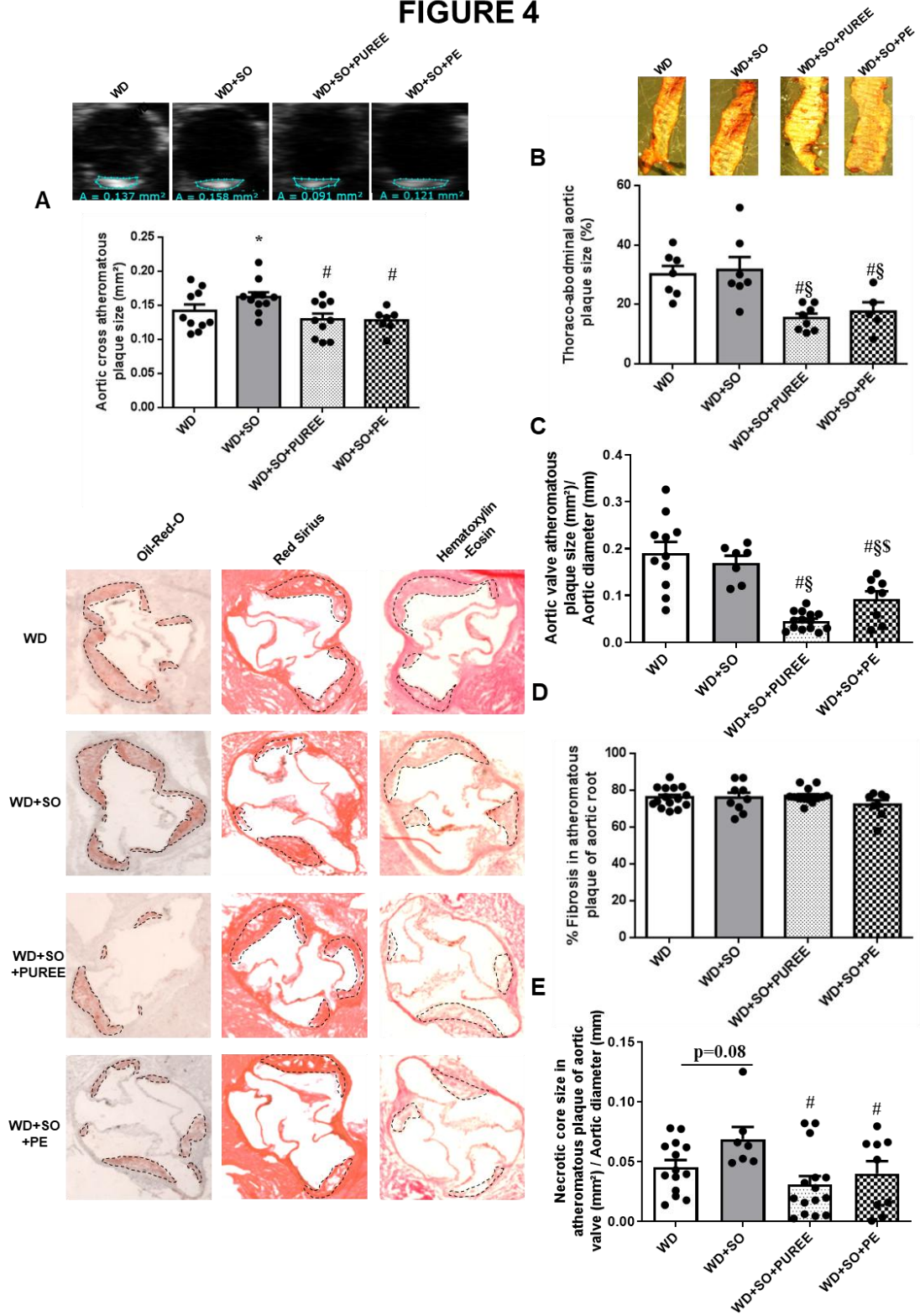
**FIGURE 3**



726

727

**FIGURE 4**

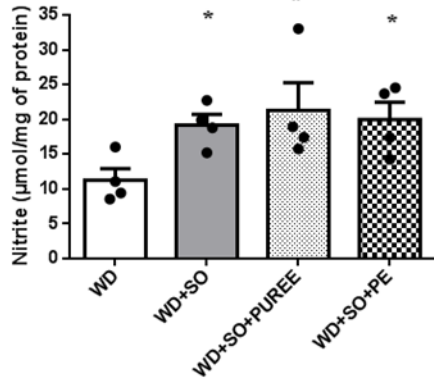


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**FIGURE 5**

**A**



**B**

