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# Effects of Cyclic Fatty Acid Monomers from Heated Vegetable Oil on Markers of Inflammation and Oxidative Stress in Male Wistar Rats

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**ABSTRACT:** This study assesses the effects of cyclic fatty acid monomers (CFAM) from heated vegetable oils on oxidative stress and inflammation. Wistar rats were fed either of these four diets for 28 days: canola oil (CO), canola oil and 0.5% CFAM (CC), soybean oil (SO), and soybean oil and 0.5% CFAM (SC). Markers of oxidative stress and inflammation were determined by micro liquid chromatography tandem mass spectrometry (micro-LC–MS/MS) and enzyme-linked immunosorbent assay (ELISA) kits, respectively. Analysis of variance (ANOVA) for a 2 × 2 factorial design was performed to determine the CFAM and oil effects and interactions between these two factors at  $P \leq 0.05$ . For significant interactions, a post hoc multiple comparison test was performed, i.e., Tukey HSD (honest significant difference) test. CFAM induced higher plasma levels of 15-F<sub>2t</sub>-IsoP (CC, 396 ± 43 ng/mL, SC, 465 ± 75 ng/mL vs CO, 261 ± 23 ng/mL and SO, 288 ± 35 ng/mL,  $P < 0.05$ ). Rats fed the SC diet had higher plasma 2,3-dinor-15-F<sub>2t</sub>-IsoP (SC, 145 ± 9 ng/mL vs CC, 84 ± 8 ng/mL, CO, 12 ± 1 ng/mL, and SO, 12 ± 1 ng/mL,  $P < 0.05$ ), urinary 2,3-dinor-15-F<sub>2t</sub>-IsoP (SC, 117 ± 12 ng/mL vs CC, 67 ± 13 ng/mL, CO, 15 ± 2 ng/mL, and SO, 18 ± 4 ng/mL,  $P < 0.05$ ), and plasma IL-6 (SC, 57 ± 10 pg/mL vs CC, 48 ± 11 pg/mL, CO, 46 ± 9 pg/mL, and SO, 44 ± 4 pg/mL,  $P < 0.05$ ) than the other three diet groups. These results indicate that CFAM increased the levels of markers of oxidative stress, and those effects are exacerbated by a CFAM–high-linoleic acid diet.

**KEYWORDS:** cyclic fatty acid monomers, soybean oil, canola oil, inflammation, oxidative stress

## 1. INTRODUCTION

Deep frying affects the quality of vegetable oils due to various chemical reaction products it generates,<sup>1,2</sup> some of which may be undesired toxic compounds.<sup>3</sup> The effects of some of the generated compounds have been studied. Mice fed a high-fat diet with polar compounds from deep-frying of palm oil displayed impaired liver function, lipid metabolism, and glucose tolerance.<sup>4</sup> Cyclic fatty acid monomers (CFAM), present at relatively low levels ranging from 100 to 6600 mg per 100 g of total fatty acids in commercial frying oils,<sup>5–7</sup> increase liver triacylglycerols (TAG) and alter fatty acid synthesis and oxidation.<sup>8,9</sup> Liver TAG accumulation is a complex phenomenon,<sup>10</sup> as liver TAG synthesis can be protective<sup>11</sup> but can also trigger lipotoxicity and induce oxidative stress and inflammation.<sup>12,13</sup> However, no study has endeavored to evaluate the relationship between dietary CFAM and oxidative stress and inflammation.

F<sub>2</sub>-isoprostanes (F<sub>2</sub>-IsoP) are a class of prostaglandin-like compounds that are generated from arachidonic acid through nonenzymatic pathways, and they are considered the gold-standard marker for the quantification of in vivo oxidative stress in humans.<sup>14</sup> Although F<sub>2</sub>-IsoP levels are not easily affected by dietary lipids,<sup>15</sup> some compounds such as cocoa extract<sup>16</sup> and some dietary fatty acids do affect the formation and excretion of F<sub>2</sub>-IsoP. Replacing a diet rich in saturated fatty

acids (SFA) with linoleic acid,<sup>17</sup> as well as replacing oleic acid with vaccenic acid,<sup>18</sup> resulted in increased levels of F<sub>2</sub>-IsoP. In premenopausal women, a significant inverse association between levels of F<sub>2</sub>-IsoP and long-chain n-3 fatty acids was found, but trans fats were positively associated with F<sub>2</sub>-IsoP.<sup>19</sup> A study among midlife women<sup>20</sup> found that SFA, linoleic, and oleic acids are associated with high levels of markers of oxidative stress. Beside F<sub>2</sub>-IsoP, other oxidized lipids have been identified, such as F<sub>4c</sub>-neuroprostanes (NeuroP), which are generated from nonenzymatic oxidation of docosahexaenoic acid (DHA) and have been implicated in neurodegenerative conditions.<sup>21</sup> However, there is no study documenting the effects of CFAM from heated vegetable oils on markers of oxidative stress using liquid chromatography tandem mass spectrometry (LC–MS/MS).

The present study was designed to determine whether CFAM added to either canola oil (high-oleic) diet or soybean oil (high-linoleic) diet can increase the risk of oxidative stress in rats. In view of this objective, we evaluated concentrations of various markers of oxidative stress in the liver, plasma, and

urine of an experimental dietary rat model and the concentrations of interleukin-6 (IL-6) and C-reactive protein (CRP), physiological markers of subclinical systemic inflammation in the plasma.<sup>22</sup>

## 2. MATERIALS AND METHODS

**2.1. Chemicals.** Methanol, acetonitrile, and chloroform (respectively, MeOH, ACN LC-MS grade, and CHCl<sub>3</sub> HPLC grade) were purchased from Fisher Scientific U.K. (Loughborough, U.K.). Hexane HPLC grade and ethanol absolute LC-MS grade were purchased from Sigma-Aldrich (Saint Quentin Fallavier, France), whereas ethyl acetate (HPLC grade) was obtained from VWR (EC). Water (H<sub>2</sub>O) and isopropyl alcohol used in this study were of LC-MS grade. KOH, formic acid, and NH<sub>4</sub>OH 28% were used in our experiments.

**2.2. Standards.** Standards used in this study are already described in a previous study by Dupuy et al.<sup>23</sup> All the following standards were synthesized according to our published procedures:<sup>24,25</sup> *ent*-16(*RS*)-9-*epi*-ST- $\Delta^{14}$ -10-PhytoF, *ent*-9(*RS*)-12-*epi*-ST- $\Delta^{14}$ -13-PhytoF, *ent*-16(*RS*)-13-*epi*-ST- $\Delta^{14}$ -9-PhytoF, *ent*-16-F<sub>11</sub>-PhytoP, *ent*-16-*epi*-16-F<sub>11</sub>-PhytoP, 9-F<sub>11</sub>-PhytoP, 9-*epi*-9-F<sub>11</sub>-PhytoP, *ent*-16-B<sub>11</sub>-PhytoP, *ent*-9-L<sub>11</sub>-PhytoP, 16(*RS*)-16-A<sub>11</sub>-PhytoP, 2,3-dinor-15-F<sub>21</sub>-IsoP, 2,3-dinor-15-*epi*-15-F<sub>21</sub>-IsoP, *ent*-2,3-dinor-5,6-dihydro-dinor-15-F<sub>21</sub>-IsoP, 15-F<sub>21</sub>-IsoP, 15-*epi*-15-F<sub>21</sub>-IsoP, 5-F<sub>21</sub>-IsoP, 10-F<sub>41</sub>-NeuroP, 10-*epi*-10-F<sub>41</sub>-NeuroP, and 4(*RS*)-4-F<sub>41</sub>-NeuroP. Just as for our internal standards, these standards' purities were assessed by high-performance liquid chromatography (HPLC) and contained <5% impurity.

**2.3. Internal Standards.** Of the three internal standards, D<sub>4</sub>-15-F<sub>21</sub>-IsoP ( $\geq 98\%$  purity) was purchased from Cayman Chemicals (Ann Arbor, MI, U.S.A.) and C21-15-F<sub>21</sub>-IsoP and D<sub>4</sub>-*epi*-10-F<sub>41</sub>-NeuroP were synthesized according to our published procedures,<sup>24,25</sup> and both had less than 5% impurity content as confirmed by our HPLC analysis.

**2.4. Animals and Diets.** Thirty-six (36) male Wistar rats initially weighing ca. 150 g were acclimated to their new environment for 7 days and fed a rat chow (Purina Canada, Mississauga, ON). After acclimation, rats were housed in individual cages and randomly assigned one of the four diets groups and had a 7 day adaptation period followed by a 28 day experimental period. Twenty days through the experiment, rats were put in metabolic cages, and urine was collected every 6 h for 24 h in tubes that were immersed in ice. All the collections of the day were pooled, 50  $\mu$ L of butylated hydroxytoluene (BHT) 0.005% in methanol was added, and they were stored first at  $-20^\circ\text{C}$  and thereafter at  $-80^\circ\text{C}$  until analysis. At the end of the 28 day period, all animals were sacrificed by cardiac puncture under isoflurane USP (Pharmaceutical Partners Canada, Richmond Hill, ON), and plasma was recovered from whole blood after centrifugation (2000 rpm, 10 min, at  $4^\circ\text{C}$ ) and stored immediately in liquid nitrogen and further at  $-80^\circ\text{C}$  until analysis.

Prior to the formulation of diets, CFAM were isolated from linseed oil heated at  $275^\circ\text{C}$  for 12 h as previously described.<sup>26</sup> Four purified diets were constituted as a combination of source of dietary fat (10% canola oil or soybean oil) and the addition of CFAM (0.0% or 0.5% CFAM of total dietary fat): canola oil (CO), canola oil and CFAM (CC), soybean oil (SO), and soybean and CFAM (SC). Each of the four diets contained 18% protein (casein), 20% sucrose, 42% maize starch, 5% cellulose, 0.3% L-cysteine, 0.2% choline bitartrate, 1% vitamin mix (AIN-93-VX0), and 3.5% mineral mix (AIN-93-MX). The four diets were isoenergetic (CO, 19.5 kJ/g; CC, 19.4 kJ/g; SO, 19.5 kJ/g; SC, 19.5 kJ/g), isolipidic [CO, 19.3% (w/w); CC, 19.3% (w/w); SO, 18.9% (w/w); SC, 19.7% (w/w)], and isonitrogenous [CO, 10.1% (w/w); CC, 10.3% (w/w); SO, 10.0% (w/w); SC, 10.2% (w/w)]. Canola oil and soybean oil fatty acid profiles were as follows, respectively (wt % of total fatty acids): saturated fatty acids or SFA (4.8 and 13.2), monounsaturated fatty acids or MUFA (61.3 and 20.5), linoleic acid or 18:2n-6 (17.8 and 52),  $\alpha$ -linolenic acid or 18:3n-3 (8 and 6.7). The ratio of n-6 to n-3 was 2.2 for canola oil and 7.8 for soybean oil.

Treatment procedures and animal care were approved by the Laval University Animal Care Committee in accordance with the Canadian Council on Animal Care guidelines (file no. 2015-004).

**2.5. Plasma Fatty Acid Analysis.** Prior to fatty acid analysis, total lipids were extracted from 200  $\mu$ L of plasma according to Folch et al.,<sup>27</sup> and fatty acids were analyzed with a gas chromatograph (Shimadzu GC-2010, Santa Clara, CA, U.S.A.) according to our methods as previously described.<sup>28</sup>

**2.6. Extraction, Analysis, and Quantification of Non-enzymatic Lipid Products.** For the analysis of F<sub>2</sub>-IsoP, we use LC-MS/MS, which is the most appropriate technique to determine F<sub>2</sub>-IsoP levels as immunoassay can generate artifactual issues.<sup>29</sup> In all the extraction processes we used Oasis Max (30  $\mu$ m, 60 mg) extraction plates (Waters, Milford, MA, U.S.A.) which were found to be suitable for LC-MS/MS per Dupuy et al.<sup>23</sup>

**2.6.1. Extraction from Urine.** **2.6.1.1. Sample Preparation.** An amount of 1 mL of urine is placed in a Pyrex tube with 1 mL of formic acid 40 mM (pH 4.5) to homogenize the pH of the sample and 5  $\mu$ L of internal standards (5 ng). The mix is then vortexed.

**2.6.1.2. Isoprostanes Extraction.** The extraction begins with the conditioning of the solid-phase extraction (SPE) column with  $2 \times 1$  mL of methanol and  $2 \times 1$  mL of formic acid 20 mM. After the column is conditioned, samples are charged on the column and the column is cleaned with  $2 \times 1$  mL of, respectively, NH<sub>3</sub> 2%, methanol/formic acid 20 mM (30:70, v/v), hexane, and hexane/ethyl acetate (70:30, v/v). Isoprostanes are eluted with hexane/ethanol/acetic acid (70:29.4:0.6, v/v/v). The solvent is then dried under a stream of nitrogen at  $40^\circ\text{C}$  for 30 min, and 100  $\mu$ L of mobile phase (water/acetonitrile, 83:17, v/v, with each containing 0.1% formic acid) is added, the sample is vortexed, left for 15 min, vortexed again, and 80  $\mu$ L is placed in a vial for micro-LC-MS/MS analysis.

**2.6.2. Extraction from Plasma.** Dupuy et al. noted that  $\geq 200$   $\mu$ L of mouse plasma was enough for the analysis of few isoprostanes.<sup>23</sup> To get a broader profile of the nonenzymatic oxidized lipid products, we used 400  $\mu$ L of plasma. Prior to the isoprostanes extraction process, 400  $\mu$ L of rat plasma was placed in tubes for hydrolysis with 20  $\mu$ L of BHT 0.005% in methanol, 950  $\mu$ L of KOH 1 M in H<sub>2</sub>O, and 5  $\mu$ L of internal standards (1 ng/ $\mu$ L). The tubes were mounted on a mixer with a programmable mixing cycle (PTR-30, Grant-bio), and then placed in an incubator (IKA KS 4000i control) for 30 min at  $40^\circ\text{C}$ . After incubation, 1 mL of formic acid 40 mM was added to each tube. Isoprostanes were then extracted as described in section 2.6.1.2.

**2.6.3. Extraction from Liver.** **2.6.3.1. Lipid Extraction.** Prostanoids were extracted from ca. 200 mg of liver in grinding matrix tubes (Lysing matrix D, MP Biochemicals, Illkirch, France) with 50  $\mu$ L of BHT 1% in methanol and 1 mL of methanol/water EGTA 5 mM (2:1). Tubes were then placed in a FastPrep-24 (MP Biochemicals), and tissues were ground for 30 s at a speed of 6.5 m/s. Lipids were extracted as previously described.<sup>27</sup> The ground tissue was transferred in centrifuge polypropylene tubes with 5 mL of chloroform/methanol (2:1), 1 mL of NaCl 0.9%, and 5  $\mu$ L of internal standards (1 ng/ $\mu$ L), and the mixture was vortexed and centrifuged at 3500 rpm for 10 min at room temperature. The organic phase was recovered in new Pyrex tubes, and the solvent was dried under a stream of nitrogen at  $40^\circ\text{C}$  for about an hour and half.

**2.6.4. Hydrolysis.** Lipids were hydrolyzed with 1 mL of KOH 1 M in methanol, in the same conditions as for plasma lipids. After hydrolysis, 2 mL of formic acid 40 mM was added, and isoprostanes were extracted as described in section 2.6.1.2.

**2.7. Analysis of Nonenzymatic Lipid Products by Micro-LC-MS/MS.** **2.7.1. LC Parameters.** The analysis was conducted on an Eksigent (Sciex Applied Biosystems, Framingham, MA, U.S.A.) micro-HPLC equipped with CTC Analytics AG (Zwingen, Switzerland) and coupled to a QTRAP 5500 system (AB SCIEX, Concord, ON, Canada). A HALO C18 column (10 mm  $\times$  0.5 mm, 2.7  $\mu$ m, Eksigent, Dublin, CA, U.S.A.) was used for the micro-HPLC analysis. The LC mobile phase consisted of a mixture of solvents A (H<sub>2</sub>O added with 0.1% formic acid) and B (ACN/MeOH, 80:20, v/v added with 0.1% formic acid) delivered at 0.03 mL $\cdot$ min<sup>-1</sup>. The binary gradient is as follows: at time 0, 83% A and 17% B; at 9.5 min, 78% A and 22% B; at

**Table 1. Standards Calibration Curves**

compd	linear regression	r	IS <sup>a</sup>
ent-16(RS)-9-epi-ST- $\Delta^{14}$ -10-PhytoF	Y = 3.08779x	0.99988	D <sub>4</sub> -10-F <sub>4t</sub> -NeuroP
ent-9(RS)-12-epi-ST- $\Delta^{14}$ -13-PhytoF	Y = 0.98618x	0.99983	D <sub>4</sub> -10-F <sub>4t</sub> -NeuroP
ent-16(RS)-13-epi-ST- $\Delta^{14}$ -9-PhytoF	Y = 3.07013x	0.99994	D <sub>4</sub> -10-F <sub>4t</sub> -NeuroP
ent-16-F <sub>1t</sub> -PhytoP	Y = 0.18298x	0.99982	D <sub>4</sub> -10-F <sub>4t</sub> -NeuroP
ent-16-epi-16-F <sub>1t</sub> -PhytoP	Y = 0.22860x	0.99985	D <sub>4</sub> -10-F <sub>4t</sub> -NeuroP
9-F <sub>1t</sub> -PhytoP	Y = 1.07221x	0.99977	D <sub>4</sub> -10-F <sub>4t</sub> -NeuroP
9-epi-9-F <sub>1t</sub> -PhytoP	Y = 1.30677x	0.99976	D <sub>4</sub> -10-F <sub>4t</sub> -NeuroP
ent-16-B <sub>1t</sub> -PhytoP	Y = 2.94248x	0.99894	D <sub>4</sub> -10-F <sub>4t</sub> -NeuroP
ent-9-L <sub>1t</sub> -PhytoP	Y = 3.21413x	0.99931	D <sub>4</sub> -10-F <sub>4t</sub> -NeuroP
16(RS)-16-A <sub>1t</sub> -PhytoP	Y = 0.49741x	0.99970	D <sub>4</sub> -10-F <sub>4t</sub> -NeuroP
2,3-dinor-15-F <sub>2t</sub> -IsoP	Y = 1.15680x	0.99991	D <sub>4</sub> -10-F <sub>4t</sub> -NeuroP
2,3-dinor-15-epi-15-F <sub>2t</sub> -IsoP	Y = 0.90891x	0.99972	D <sub>4</sub> -10-F <sub>4t</sub> -NeuroP
ent-2,3-dinor-5,6-dihydro-dinor-15-F <sub>2t</sub> -IsoP	Y = 3.78958x	0.99974	D <sub>4</sub> -10-F <sub>4t</sub> -NeuroP
15-F <sub>2t</sub> -IsoP	Y = 0.24073x	0.99868	D <sub>4</sub> -10-F <sub>4t</sub> -NeuroP
15-epi-15-F <sub>2t</sub> -IsoP	Y = 0.35380x	0.99983	D <sub>4</sub> -10-F <sub>4t</sub> -NeuroP
5-F <sub>2t</sub> -IsoP	Y = 0.21236x	0.99977	D <sub>4</sub> -10-F <sub>4t</sub> -NeuroP
10-F <sub>4t</sub> -NeuroP	Y = 0.96412x	0.99985	D <sub>4</sub> -10-F <sub>4t</sub> -NeuroP
10-epi-10F <sub>4t</sub> -NeuroP	Y = 0.21099x	0.99968	D <sub>4</sub> -10-F <sub>4t</sub> -NeuroP
4(RS)-4-F <sub>4t</sub> -NeuroP	Y = 0.08500x	0.99816	D <sub>4</sub> -10-F <sub>4t</sub> -NeuroP

<sup>a</sup>IS, internal standard.

11.5 min, 70% A and 30% B; at 15 min, 70% A and 30% B, and then at 16 min, 5% A and 95% B for 2.3 min, for a total run time of 21 min. The sample injection volume was 5  $\mu$ L.

**2.7.2. MS/MS Conditions.** The MS/MS was operated in the electrospray ionization (ESI) negative mode with a source voltage kept at -4.5 kV and N<sub>2</sub> used as curtain gas. For each analyte, the collision energy was optimized to maximize the ion currents of the precursor to produce ion dissociation. The analytes were detected by MS/MS using multiple reaction monitoring (MRM). All the MRM for our analytes have already been reported by Dupuis et al.<sup>23</sup>

**2.7.3. Quantification of Nonenzymatic Lipid Products.** The quantitation of prostanoids was done with MultiQuant 3.0 software (AB SCIEX). Prior to the quantification, calibration curves were obtained from the ratio of the areas under the curves of isoprostanes and those of the internal standard (IS) as a function of the ratio of the concentration of isoprostanes and IS. The linear regressions obtained for all the standards are presented in Table 1.

**2.7.4. Extraction Yield and Matrix Effect.** The yield gives the percentage of any given analyte recovered after SPE, while matrix effect refers to the difference in mass spectrometric response for analyte in standard solution versus the response of the same analyte in a biological matrix. Two concentrations of standards were used to calculate these two parameters: 32 pg/ $\mu$ L (C32) and 256 pg/ $\mu$ L (C256). For each concentration, duplicate samples were prepared before and after column extraction and a standard solution into starting mobile phase was also prepared and used as quality control. The yield for each concentration corresponds to the ratio between the area under the curve (AUC) of the analyte before and after extraction. The matrix effect is the ratio, for each concentration, between the AUC of any analyte after extraction and the AUC of the standard solution.

**2.8. Statistical Analysis.** All the results are expressed as mean  $\pm$  SD ( $n = 9$ ). Analysis of variance (ANOVA) for 2  $\times$  2 factorial experimental design was performed with SAS software (SAS Institute Inc., Cary, NC, U.S.A.) to determine the CFAM and oil effects, as well as interactions among CFAM and dietary oils. For plasma IL-6, a logarithmic transformation of data was applied [ $\text{Log}_{10}(x + 1)$ ] to achieve the homogeneity of variance. When significant interactions were observed between oil and CFAM, a multiple comparison test, i.e., Tukey honest significant difference (HSD) test, was used to determine differences between the groups. Pearson correlation coefficients were calculated between variables. The level of significance was set at  $P \leq 0.05$ .

### 3. RESULTS

#### 3.1. Yield, Matrix Effect, and Repeatability and Precision of the Method.

Yields and matrix effects are expressed as mean in %  $\pm$  SD % and presented in Table 2.

**Table 2. Summary Data for Isoprostanes and Neuroprostanes Yield and Matrix Effect**

compd <sup>a</sup>	yield		matrix effect	
	mean (%)	SD%	mean (%)	SD%
	Liver			
D <sub>4</sub> -15-F <sub>2t</sub> -IsoP (IS)	84	9	75	2
D <sub>4</sub> -10-epi-F <sub>4t</sub> -NeuroP (IS)	64	18	72	1
15-F <sub>2t</sub> -IsoP	100	5	85	2
2,3-dinor-15-F <sub>2t</sub> -IsoP	105	9	73	2
4(RS)-F <sub>4t</sub> -NeuroP	103	8	56	7
	Plasma			
D <sub>4</sub> -15-F <sub>2t</sub> -IsoP (IS)	75	8	59	5
D <sub>4</sub> -10-epi-F <sub>4t</sub> -NeuroP (IS)	100	7	70	4
15-F <sub>2t</sub> -IsoP	79	5	68	3
2,3-dinor-15-F <sub>2t</sub> -IsoP	33	6	82	2
4(RS)-F <sub>4t</sub> -NeuroP	91	7	69	3
	Urine			
D <sub>4</sub> -15-F <sub>2t</sub> -IsoP (IS)	90	4	104	9
D <sub>4</sub> -10-epi-F <sub>4t</sub> -NeuroP (IS)	90	3	69	6
15-F <sub>2t</sub> -IsoP	99	7	77	6
2,3-dinor-15-F <sub>2t</sub> -IsoP	94	5	77	4
4(RS)-F <sub>4t</sub> -NeuroP	101	7	66	7

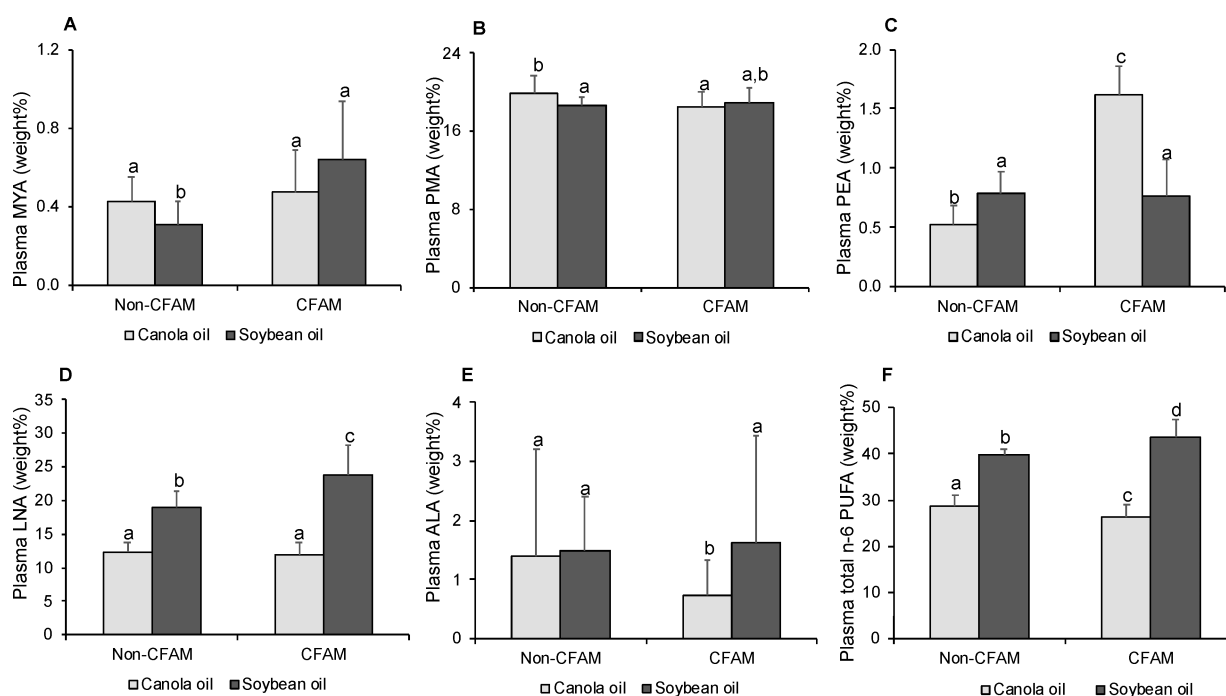
<sup>a</sup>IS, internal standard; IsoP, isoprostanes; NeuroP, neuroprostanes.

Extractions from urine had the highest yields and lowest variation compared to extractions from liver and plasma, suggesting a putative impact of the hydrolysis step on the extraction process. In order to determine the linear range in the quantification process, we used 15 concentrations ranging from  $3.125 \times 10^{-3}$  to 512 pg/ $\mu$ L<sup>-1</sup> prepared in triplicate and injected. This allowed us to establish calibration curves and calculating the linear regression equation. The detector response was linear across the range tested. Limit of detection

**Table 3. Fatty Acid Composition (wt %) in Total Plasma Lipids of Rats Fed Diets Containing Canola Oil (CO), Canola Oil and CFAM (CC), Soybean Oil (SO), and Soybean Oil and CFAM (SC)<sup>a</sup>**

fatty acid <sup>b</sup>	diet				P values	
	CO	CC	SO	SC	oil	CFAM <sup>c</sup>
16:1 9c	1.8 ± 0.3	2.3 ± 0.3	1.7 ± 0.3	1.4 ± 1.2	NS	NS
18:0	8.8 ± 0.9	10.0 ± 1.5	9.2 ± 1.2	9.0 ± 1.8	NS	NS
18:1(n-9)	23.7 ± 2	23.9 ± 1.8	9.3 ± 1.8	10.7 ± 2.0	**	NS
18:1(n-7)	2.0 ± 0.3	2.4 ± 0.6	1.9 ± 0.3	1.7 ± 0.6	*	NS
20:1(n-9)	3.4 ± 0.6	3.0 ± 0.6	2.9 ± 0.6	2.9 ± 1.2	*	NS
20:4(n-6)	16.5 ± 3.0	14.4 ± 1.8	20.9 ± 2.7	20.0 ± 4.8	*	NS
20:5(n-3)	1.4 ± 0.6	1.4 ± 0.6	0.8 ± 0.18	0.9 ± 0.3	*	NS
22:5(n-6)	2.4 ± 0.6	1.5 ± 0.6	2.8 ± 0.9	1.8 ± 0.9	NS	*
22:6(n-3)	3.2 ± 1.2	2.5 ± 0.6	5.3 ± 1.8	3.5 ± 1.8	NS	*
∑SFA <sup>d</sup>	29.1 ± 1.8	29.0 ± 2.1	28.1 ± 1.8	29.6 ± 2.4	NS	NS
∑MUFA <sup>e</sup>	31.4 ± 3.0	33.3 ± 3.0	16.6 ± 2.1	17.4 ± 2.7	**	NS
∑PUFA n-3 <sup>f</sup>	8.4 ± 1.8	6.2 ± 0.9	10.4 ± 2.1	7.9 ± 2.4	NS	*
∑PUFA <sup>g</sup>	37.1 ± 3.0	32.5 ± 3.0	50.3 ± 3.0	51.6 ± 3.0	**	NS
22:5n-6/22:6n-3	0.7 ± 0.0	0.5 ± 0.0	0.5 ± 0.6	0.5 ± 0.0	NS	*

<sup>a</sup>Data are mean ± SD (ANOVA,  $P < 0.05$ ,  $n = 9$ ). \*,  $P < 0.05$ ; \*\*,  $P < 0.01$ ; NS, not significant. <sup>b</sup>Data for myristic, palmitic, palmitelaidic, linoleic, and  $\alpha$ -linolenic acids and total n-6 PUFA are presented in Figure 1. <sup>c</sup>CFAM: cyclic fatty acid monomers. <sup>d</sup>Sum of the saturated fatty acids. <sup>e</sup>Sum of the monounsaturated fatty acids. <sup>f</sup>Sum of the PUFA n-3. <sup>g</sup>Sum of the total PUFA.



**Figure 1.** Effects of oil and CFAM on plasma myristic acid (MYA, A), palmitic acid (PMA, B), palmitelaidic acid (PEA, C), linoleic acid (LNA, D),  $\alpha$ -linolenic acid (ALA, E), and total n-6 polyunsaturated fatty acids (n-6 PUFA, F) of rats fed either canola oil or soybean oil with or without 0.5% of CFAM. Data are mean ± SD. Data with different letter are significantly different [ANOVA followed by a post hoc Tukey honestly significant different (HSD) test,  $P \leq 0.05$ ,  $n = 9$ ].

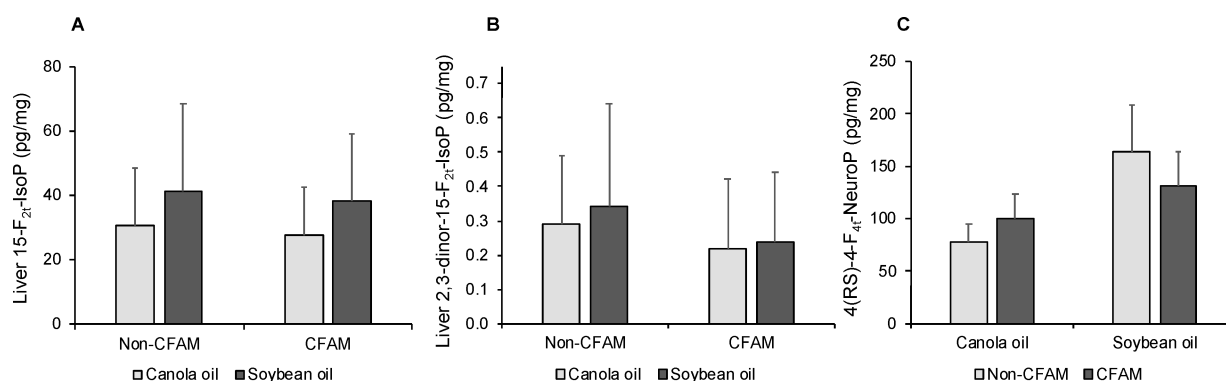
(LOD) and limit of quantitation (LOQ) were also determined and ranged, respectively, from 0.16 to 0.63 pg injected and between 0.16 and 1.25 pg injected. These values depended on the type of isoprostanoids but were quite homogeneous.

**3.2. Food Intake and Body Weight Gain.** No changes were observed between the four diet groups for daily food intake or weight gain ( $P > 0.05$ ).

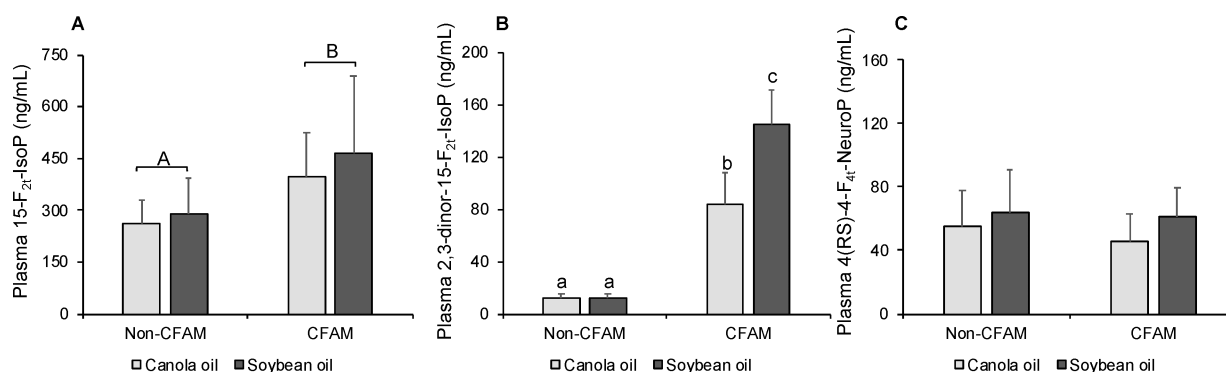
**3.3. Plasma Fatty Acid Composition.** As per data presented in Table 3, canola oil induced higher plasma levels of oleic acid, total MUFA, and eicosapentaenoic acid (EPA) than soybean oil, and rats fed soybean oil had higher levels of

plasma arachidonic acid (ARA) and total polyunsaturated fatty acids (PUFA) than canola oil ( $P < 0.05$ ). The CFAM diets induced lower levels of DPAn-6, DHA, 22:5n-6/22:6n-3 ratio, and total n-3 PUFA than the non-CFAM diets ( $P < 0.05$ ).

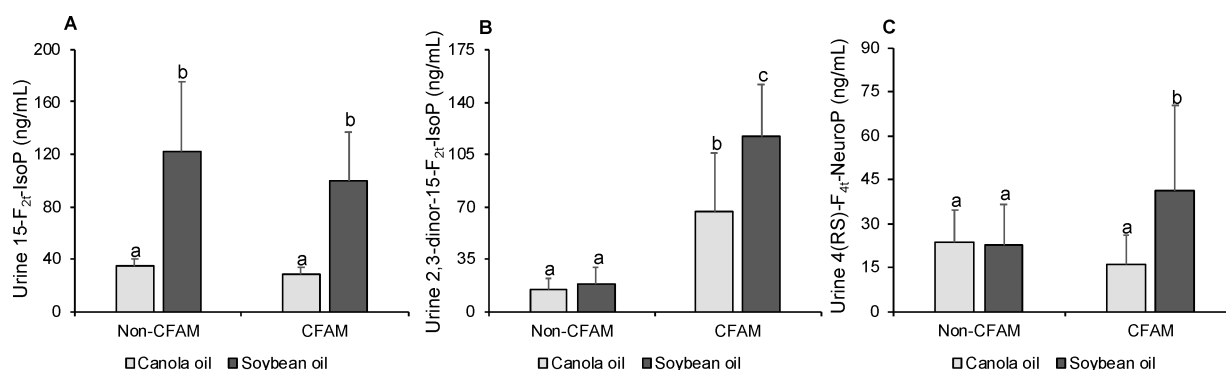
Dietary lipids modulated the effects of CFAM on six plasma fatty acids levels. Rats fed the soybean oil and CFAM (SC) diet had more linoleic acid (LNA) (Figure 1D) and PUFA n-6 (Figure 1F) than those fed the three other diets ( $P < 0.05$ ). Rats fed the soybean oil (SO) diet had more LNA (Figure 1D) and PUFA n-6 (Figure 1F) than those fed the two canola oil diets, and less myristic acid (MYA) (Figure 1A) than the three



**Figure 2.** Liver 15-F<sub>2t</sub>-IsoP (A), 2,3-dinor-15-F<sub>2t</sub>-IsoP (B), and 4(RS)-4-F<sub>4t</sub>-NeuroP (C) of rats fed either canola oil or soybean oil with or without 0.5% of CFAM. Data are mean  $\pm$  SD (ANOVA,  $P \leq 0.05$ ,  $n = 9$ ).



**Figure 3.** Plasma 15-F<sub>2t</sub>-IsoP (A), 2,3-dinor-15-F<sub>2t</sub>-IsoP (B), and 4(RS)-4-F<sub>4t</sub>-NeuroP (C) of rats fed either canola oil or soybean oil with or without 0.5% of CFAM. Data are mean  $\pm$  SD. Data with different letter superscripts are statistically different (ANOVA followed by a post hoc Tukey HSD test,  $P \leq 0.05$ ,  $n = 9$ ).



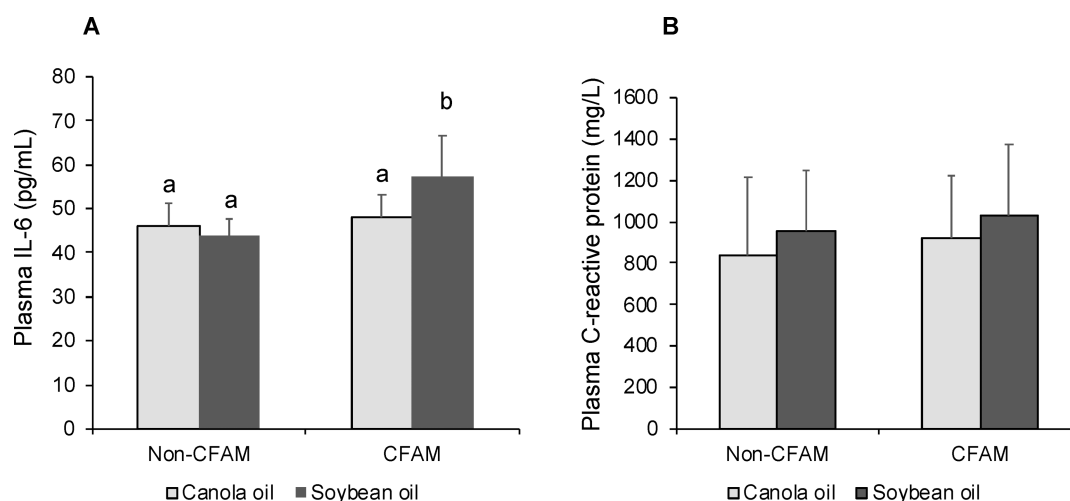
**Figure 4.** Urinary 15-F<sub>2t</sub>-IsoP (A), 2,3-dinor-15-F<sub>2t</sub>-IsoP (B), and 4(RS)-4-F<sub>4t</sub>-NeuroP (C) of rats fed either canola oil or soybean oil with or without 0.5% of CFAM. Data are mean  $\pm$  SD. Data with different letter superscripts are statistically different (ANOVA followed by a post hoc Tukey HSD  $P \leq 0.05$ ,  $n = 9$ ).

other groups ( $P < 0.05$ ). Rats fed the canola oil and CFAM diet (CC) had less  $\alpha$ -linolenic acid (ALA) (Figure 1E) and PUFAn-6 (Figure 1F) and more palmitelaidic acid (PEA) (Figure 1C) than those fed the three other diets ( $P < 0.05$ ). Rats fed the CO diet had also more palmitic acid (PMA) (Figure 1B) than those fed the SO and CC diets. Plasma PEA levels (Figure 1C) were lower in the CO group compared with the three other groups.

**3.4. Mediators of Oxidative Stress: Isoprostanes and Neuroprostanes.** **3.4.1. Liver Isoprostanes and Neuroprostanes.** Our results show that there was neither oil nor CFAM effect on liver 15-F<sub>2t</sub>-IsoP (Figure 2A) and liver 4(RS)-F<sub>4t</sub>-NeuroP (Figure 2C) ( $P > 0.05$ ). However, rats fed CFAM

tended ( $P = 0.053$ ) to have lower 2,3-dinor-15-F<sub>2t</sub>-IsoP (Figure 2B) metabolite of 15-F<sub>2t</sub>-IsoP, compared to those fed the non-CFAM diets.

**3.4.2. Plasma Isoprostanes and Neuroprostanes.** We observed a CFAM effect on plasma 15-F<sub>2t</sub>-IsoP (Figure 3A), with the CC and SC diets inducing higher levels than the CO and SO diets ( $P < 0.05$ ). There was a statistical interaction between oil and CFAM on plasma 2,3-dinor-15-F<sub>2t</sub>-IsoP (Figure 3B), with rats fed the SC diet having more than the other three diet groups ( $P < 0.05$ ) and rats fed the CC diet inducing more than those fed the CO and SO diets ( $P < 0.05$ ). There was neither oil nor CFAM effect on plasma 4(RS)-F<sub>4t</sub>-NeuroP ( $P > 0.05$ ).



**Figure 5.** Plasma IL-6 and CRP of rats fed either canola oil or soybean oil with or without 0.5% of CFAM. Data are mean  $\pm$  SD. Data with different letter superscripts are statistically different (ANOVA followed by the post hoc Tukey HSD test,  $P \leq 0.05$ ,  $n = 9$ ).

**3.4.3. Urinary Isoprostanes and Neuroprostanes.** We observed an oil effect on urinary 15-F<sub>2t</sub>-IsoP (Figure 4A), rats fed soybean oil having higher levels than those fed canola oil ( $P < 0.05$ ). We observed a statistical interaction between oil and CFAM on 2,3-dinor-15-F<sub>2t</sub>-IsoP (Figure 4B), showing that rats fed soybean oil and CFAM had more than those fed the three other diets and rats fed the canola oil and CFAM diet inducing more than those fed the CO and SO diets ( $p < 0.05$ ). There was also a significant statistical interaction between oil and CFAM on 4(RS)-F<sub>4t</sub>-NeuroP (Figure 4C): rats fed the soybean oil and CFAM diet had higher levels compared to those fed one of the other three diet groups.

**3.5. Inflammatory Markers.** There was a significant statistical interaction between oil and CFAM on plasma IL-6 (Figure 5A), as rats fed soybean oil and CFAM had higher plasma levels compared to those fed either one of the other three diet groups ( $P < 0.05$ ). We found no difference for plasma CRP (Figure 5B).

## 4. DISCUSSION

The present study aimed to assess the effects of CFAM on markers of inflammation and oxidative stress and to evaluate whether those effects can be modulated by dietary lipids. Our results showed that rats fed the soybean oil and CFAM (SC) diet had higher concentrations of LNA, PUFA<sub>n-6</sub>, and IL-6 in plasma as well as higher 2,3-dinor-15F<sub>2t</sub>-IsoP in plasma and urine and higher 4(RS)-F<sub>4t</sub>-NeuroP in urine than those fed the three other diets ( $P < 0.05$ ). To the best of our knowledge, this study is the first study to report deleterious effects of CFAM on markers of oxidative stress and inflammation when incorporated in a high-linoleic acid diet in rats.

**4.1. Changes in Plasma Fatty Acids.** Changes observed in plasma fatty acids among the four diet groups resulted either from oil and/or CFAM effect (Table 3) or from interactions between oil and CFAM (Figure 1). The high levels of linoleic acid (LNA, 18:2n-6) and n-6/n-3 ratio (2.2 and 7.8, respectively, for dietary canola oil and soybean oil) may account for the high plasma n-6/n-3 ratio and high concentrations of LNA and ARA and total n-6 PUFA found in rats fed soybean oil diet with or without CFAM. Dietary LNA can further be metabolized in many tissues by a series of desaturation and elongation steps to particularly ARA, and

possibly docosapentaenoic acid (DPAn-6, 22:5n-6). These fatty acids serve as components of membrane structural lipids and are immediate precursors to eicosanoids and isoprostanes via enzymatic and nonenzymatic pathways, respectively. In addition, ARA is the most abundant PUFA in neural tissue and in the brain,<sup>30,31</sup> and the role of ARA as an eicosanoid precursor is major in a variety of immune and inflammatory responses.<sup>32</sup> High proportions of oleic acid found in plasma of rats fed canola oil compared to those fed soybean oil can have a dietary cause since canola oil diets contained roughly 3 times more oleic acid than soybean oil (61.3 wt % of total fatty acids for canola oil vs 20.5 for soybean oil). These results show that dietary lipids affect circulating fatty acid levels as previously reported.<sup>33</sup>

DPAn-6 and DHA are, respectively, the end products in the biosynthetic pathways of the n-6 and n-3 fatty acids, respectively, from LNA and  $\alpha$ -linolenic acid, and fatty acid desaturases (FADS), i.e.,  $\Delta$ -6 and  $\Delta$ -5, are rate-limiting in those pathways.<sup>34</sup> The lower levels of DPAn-6, DHA, and DPAn-6/DHA ratio in the plasma of rats fed the CFAM diets suggest some alteration of the biosynthetic pathways of those two fatty acids. It has been demonstrated that  $\Delta$ -6 desaturase (FADS2) is the rate-limiting enzyme for the conversion of linoleic acid and  $\alpha$ -linolenic acid to, respectively,  $\gamma$ -linolenic acid (18:3n-6) and stearidonic acid (18:4n-3).<sup>35,36</sup> In fact, it has been found that trans fatty acids (TFA) and high cholesterol levels, among other factors, impair the activity of FADS2. Lower plasma levels of DPAn-6 and DHA observed in this study suggest some similarities between CFAM and other products of thermo-oxidation of vegetable oils such as TFA on the biosynthetic pathways leading to DPA and DHA. Interestingly, there were 46.3% trans isomers in our CFAM fraction, but this study cannot determine whether the above effects were solely due to those trans isomers.

**4.2. Soybean Oil Induces More Urinary 15-F<sub>2t</sub>-IsoP than Canola Oil.** The observation that soybean oil induced more urinary 15-F<sub>2t</sub>-IsoP, a marker of oxidative stress, than canola oil is consistent with literature. Although isoprostanes were not measured in their study, Rom et al.<sup>37</sup> found that soybean oil can induce oxidative stress by increasing lipid peroxides and stimulating macrophage foam cell formation. Our results are also in accord with those found by Gustafsson

**Table 4. Significant Correlations between Plasma IL-6, Fatty Acids, and Plasma and Urinary Isoprostanes ( $P < 0.05$ )<sup>a</sup>**

	plasma 15-F <sub>2t</sub> -IsoP	plasma 2,3-dinor-15-F <sub>2t</sub> -IsoP	urinary 15-F <sub>2t</sub> -IsoP	urinary 2,3-dinor-15-F <sub>2t</sub> -IsoP	urinary 4(RS)-F <sub>4t</sub> -NeuroP
IL-6	$r = 0.47$ $P = 0.004$	$r = 0.45$ $P = 0.0062$	–	$r = 0.47$ $P = 0.0038$	$r = 0.43$ $P = 0.009$
oleic acid	–	–	$r = -0.69$ $P < 0.0001$	–	$r = -0.46$ $P = 0.005$
linoleic acid	–	–	$r = 0.51$ $P = 0.0015$	–	$r = 0.39$ $P = 0.02$
arachidonic acid	–	–	$r = 0.50$ $P = 0.002$	–	$r = 0.44$ $P = 0.007$

<sup>a</sup> $r$  = Pearson correlations coefficients; –, nonsignificant correlations.

et al.<sup>38</sup> who observed that, compared to soybean oil, canola oil increased  $\gamma$ -tocopherol, an antioxidant, in serum of hyperlipidemic subjects. Moreover, it has also been found that intakes of omega-6 polyunsaturated fatty acids (n-6 PUFA) higher than the median was associated with increased serum level of 8-isoprostane F<sub>2 $\alpha$</sub>  (15-F<sub>2t</sub>-IsoP) in subjects with type 2 diabetes mellitus with certain ApoA2 genotypes.<sup>39</sup> Furthermore, a study found that rats fed an n-6 PUFA-rich oil had significantly reduced high-density lipoprotein (HDL)-cholesterol and high lipid peroxidation compared to those fed an n-3 PUFA-rich diet.<sup>40</sup> Interestingly, we found higher levels of ARA—precursor to 15-F<sub>2t</sub>-IsoP—in rats fed soybean oil compared with those fed canola oil (Table 3), and there was a positive correlation between ARA and LNA and urinary 15-F<sub>2t</sub>-IsoP (ARA,  $r = 0.50$ ,  $P = 0.002$ ; LNA,  $r = 0.51$ ,  $P = 0.001$ ). In contrast, plasma oleic acid (OLA), the major fatty acid in our canola oil, was negatively correlated with urinary 15-F<sub>2t</sub>-IsoP ( $r = -0.69$ ,  $P < 0.0001$ ). Therefore, our results strongly suggest that soybean oil is a pro-oxidant oil as compared to canola oil.

**4.3. CFAM Diets Induce More Plasma 15-F<sub>2t</sub>-IsoP than Non-CFAM Diets.** Deep-fried oils have been reported to induce liver and serum lipid peroxidation in rats.<sup>41,42</sup> Using cultured porcine endothelial cells, Flickinger et al. observed that CFAM—which are products of heat treatment of vegetable oils—increased the production and secretion of prostacyclin (PGI<sub>2</sub>) which derives from ARA through the enzymatic cyclooxygenase pathway.<sup>43</sup> Because F<sub>2</sub>-isoprostanes derive from ARA under oxidative stress conditions, our results on plasma 15-F<sub>2t</sub>-IsoP suggest that CFAM may enhance the production of isoprostanes via a free radical nonenzymatic mechanism involving the peroxidation of ARA.

**4.4. Soybean Oil and CFAM Diet Induces Higher Levels of Plasma and Urinary 2,3-Dinor-15-F<sub>2t</sub>-IsoP, Urinary 4(RS)-4-F<sub>4t</sub>-Neuroprostanes, and Plasma IL-6 than the Other Three Diets.** The CFAM diets induced higher plasma levels of 15-F<sub>2t</sub>-IsoP than the non-CFAM diets. Furthermore, the soybean and CFAM (SC) diet induced more plasma and urinary isoprostanes and plasma IL-6 than the canola oil and CFAM (CC) diet. The elevated levels of 2,3-dinor-15-F<sub>2t</sub>-IsoP, which is the metabolite of 15-F<sub>2t</sub>-IsoP, in plasma and urine as well as 4(RS)-4-F<sub>4t</sub>-NeuroP in urine of rats fed the SC diet indicate a general oxidative status which is associated with inflammation<sup>44</sup> as evidenced by the positive correlations between plasma IL-6 and plasma and urinary 2,3-dinor-15-F<sub>2t</sub>-IsoP ( $r = 0.45$ ,  $P = 0.006$  and  $r = 0.47$ ,  $P = 0.004$ , respectively, Table 4). Thus, our results suggest that high levels of IL-6 observed in the plasma of rats fed the SC diet resulted from a combination of factors related to dietary and plasma fats: First, rats fed the SC diet had high levels of LNA, and

some oxidized forms of LNA such as 9-HODE are known to have pro-inflammatory properties.<sup>45</sup> Second, rats fed the soybean oil diets had high levels of ARA and its nonenzymatic oxidized metabolites, i.e., plasma and urinary 15-F<sub>2t</sub>-IsoP and 2,3-dinor-15-F<sub>2t</sub>-IsoP, than those fed the canola oil diets. Third, rats fed CFAM diets had lower levels of DPAn-6 and DHA (Table 3) than those fed non-CFAM diets. DPAn-6 reduces the activity of cyclooxygenase-2 (COX-2) and is a potent inhibitor of pro-inflammatory PGE<sub>2</sub> production and, when combined with DHA, DPA-n-6 enhances the anti-inflammatory activity of DHA.<sup>46,47</sup> Therefore, our results strongly suggest that CFAM amplify the pro-oxidant and pro-inflammatory effect of soybean oil and might greatly account for the high levels of 2,3-dinor-15-F<sub>2t</sub>-IsoP and IL-6 in plasma of rats fed soybean oil and CFAM diet.

Our results show that CFAM are associated with increased production of markers of oxidative stress in vivo and this effect is exacerbated when CFAM are supplemented with a n-6 PUFA-rich diet. On a nutritional level, this observation reinforces the need for a better selection of vegetable oils for frying, favoring oils with lower PUFA content.

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## ■ ABBREVIATIONS USED

CFAM, cyclic fatty acid monomers; DHA, docosahexaenoic acid; DPA, docosapentaenoic acid; LNA, linoleic acid; ARA, arachidonic acid; OLA, oleic acid; PUFA, polyunsaturated fatty acid; SFA, saturated fatty acid; TFA, trans fatty acid; IL-6, interleukin-6; CRP, C-reactive protein; IsoP, isoprostane;

NeuroP, neuroprostanes; FADS, fatty acid desaturase; CO, canola oil; CC, canola oil and CFAM; SO, soybean oil; SC, soybean oil and CFAM; LC-MS/MS, liquid chromatography tandem mass spectrometry

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