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Protective effects of n-6 fatty acids-enriched diet on intestinal ischemia/reperfusion injury involve lipoxin A4 and its receptor.

Running title: Effects of n-6 diet on ischemia/reperfusion injury

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Abbreviations:

Acetonitrile (ACN), arachidonic acid (AA), bovine serum albumin (BSA), cyclooxygenase (COX), dihydroxy-eicosatetraenoic acid (DiHETE), docosahexaenoic acid (DHA), eicosapentaenoic acid (EPA), epoxyeicosatrienoic acid (EET), formic acid (FA), formyl peptide receptor (FPR), gas chromatography (GC), Hank's balanced salt solution (HBSS), hydroxydocosahexaenoic acid (HDoHE), hydroxyeicosapentaenoic acid (HEPE), hydroxyeicosatetraenoic acid (HETE), ischemia/reperfusion (I/R), chemokine (C-X-C motif) ligand 1 (CXCL1), leukotriene B_4 (LTB_4), leukotriene B_4 deuterated (LTB_4-d4), leukotriene B_5 (LTB_5), limit of detection (LOD), limit of quantification (LOQ), lipoxin A_4 (LxA_4), lipoxin A_4 deuterated (LxA_4-d5), lipoxin B_4 (LxB_4), lipoxygenase (LOX), liquid chromatography/tandem mass spectrometry (LC-MS/MS), 7(S)-maresin (7-MaR1), methanol (MeOH), chemokine ligand 2 (CCL2), myeloperoxidase (MPO), neonatal necrotizing enterocolitis (NEC), oxoeicosatetraenoic acid (oxo-ETE), phosphate-buffered saline (PBS), polyunsaturated fatty acids (PUFA), prostaglandin A_1 (PGA_1), 8-iso prostaglandin A_2 (8-iso PGA_2), prostaglandin E_2 (PGE_2), prostaglandin E_3 (PGE_3), 6-keto-prostaglandin $F_{1\alpha}$ ($6kPGF_{1\alpha}$), 10(S),17(S)-protectin (PDx), resolvin D1 (RvD1), resolvin D2 (RvD2), superior mesenteric artery (SMA), thromboxane B_2 (TXB_2), ultra high pressure liquid chromatography (UHPLC).

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Summary

Background and purpose: Long-term intake of dietary fatty acids is known to predispose to chronic inflammation, but their potential influence on acute intestinal ischemia/reperfusion (I/R) injury is unknown. The aim of this study was to determine the consequences of a diet rich in n-3 or n-6 polyunsaturated fatty acids (PUFA) on intestinal I/R-induced damage.

Experimental approach: Mice were fed 3 different isocaloric diets: a balanced diet used as a control, and two different PUFA-enriched diets, providing either high n-3 or high n-6 intake. Intestinal injury was evaluated after intestinal I/R. PUFA metabolite quantification was performed in intestinal tissues by liquid chromatography tandem mass spectrometry.

Key results: In control diet-fed mice, intestinal I/R caused inflammation and increased cyclooxygenase (COX) and lipoxygenase (LOX)-derived metabolites, compared to sham-operated animals. Lipoxin A_4 (LxA_4) was significantly and selectively increased after ischemia. Animals fed a high n-3 diet did not display a different inflammatory profile following intestinal I/R, compared to control diet-fed animals. In contrast, intestinal inflammation was decreased in I/R group fed a high n-6 diet and level of LxA_4 was increased post-ischemia, compared to control diet-fed mice. Blockade of formyl-peptide receptor-2, the LxA_4 receptor, abrogated n-6 rich diet-associated anti-inflammatory effects.

Conclusions and Implications: This study indicates that high n-6, but not n-3, PUFAs leads to significant protection against intestinal I/R-induced damage and demonstrates that LxA4 endogenous production can be influenced by diet.

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Introduction

There is growing evidence, which indicates bioactive lipid mediators derived from n-3 or n-6 polyunsaturated fatty acid (PUFA) play a pivotal role in initiating and sustaining the inflammatory response or engaging pro-resolving/anti-inflammatory pathways in several cardiovascular and inflammatory pathologies. These include atherosclerosis, thrombosis, arrhythmia, ischemia reperfusion (I/R) injury (De Caterina, 2011). The recent development of new techniques of high sensitivity liquid chromatography-tandem mass spectrometry (LC-MS/MS) allows a better comprehension of disease-associated lipidic metaboloma detecting simultaneously the presence of a number of PUFA metabolites in tissues. In our previous study, we characterized, by LC-MS/MS, the tissue profile of n-6 and n-3 PUFA metabolites in an experimental model of murine intestinal ischemia reperfusion (I/R) (Gobbetti *et al.*, 2013). We identified temporal and quantitative differences in n-3 and n-6 PUFA metabolite production, which correlated with inflammatory damage. However, it is unclear whether modulating the n-3/n-6 ratio composition in membranes could be used as therapeutic tool to modify the resistance of the gut to I/R injury.

Acute mesenteric ischemia is a life-threatening pathological event associated with a number of diseases including vessel occlusion, hernias, septic shock, major cardiovascular surgery, necrotizing enterocolitis and small bowel transplantation (Cerqueira *et al.*, 2005; Eltzschig *et al.*, 2011; Vollmar *et al.*, 2011). Intestinal ischemia remains a clinical challenge due to difficulty in diagnosis and especially to the lack of established pharmacological treatments. Therefore, the need for new therapeutic strategies is urgent. The intestinal mucosa is particularly prone to I/R injury due to the anatomical and physiological characteristic of the villus microcirculation (Vollmar *et al.*, 2011). The temporary interruption of the blood leads to a lack

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of oxygen supply to the tissue (ischemia), which in turn causes cellular dysfunction, protease and phospholipases activation (Gobbetti *et al.*, 2012; Otamiri *et al.*, 1987; Vollmar *et al.*, 2011). Paradoxically, the restoration of blood flow and the consequent tissue re-oxygenation (reperfusion) aggravates the local (epithelial/endothelial damage) and systemic inflammatory response, leading to bacterial translocation and multiple organ failure (Cerqueira *et al.*, 2005).

The main objective of this study was to identify if the modification of n-6/n-3PUFA status induced by the modification of PUFA metabolite biosynthesis associated with diet changes might improve intestinal resistance to ischemia reperfusion injury. We performed a dietary intervention by exposing mice to a diet rich in n-3, n-6 PUFA or to a balanced diet (Ducheix *et al.*, 2013; Kelavkar *et al.*, 2006) and we evaluated the importance of n-3 and n-6 PUFA metabolites on intestinal I/R injury. Dietary intervention significantly changed the abundance of hepatic PUFA and intestinal PUFA metabolites. Intestinal inflammation was decreased in the group fed with a high n-6 diet and the level of LxA_4 after ischemia was increased compared to the group fed with a balanced diet. Early systemic pharmacological blockade of LxA_4 receptor, formyl-peptide receptor-2 (Fpr2), abrogated the anti-inflammatory effects attained by the n-6 rich diet. This study indicates that early mobilization of LxA_4 leads to significant protection against intestinal I/R-induced damage.

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MATERIALS AND METHODS

Animals

C57 BL/6 male mice (3 weeks-old) were purchased from Janvier (Le Genest Saint Isle, France). Animals were maintained in ventilated cages (4 mice per cage) in a specific pathogen free room at 20–24°C and relative humidity (40%–70%) with a 12 hours light/dark cycle and given free access to food and water. Animal Care and ethic Committee of US006/CREFE (CEEA-122) approved the whole study protocol (permit No. MP/01/64/09/12). In total 160 mice were included in this study. All animal experiments were conducted in accordance with Guide for the Care and Use of Laboratory Animals of the European council, and were reported in accordance with the ARRIVE guidelines (Kilkenny *et al.*, 2010).

Chemicals

N-tert-butoxycarbonyl-L-Phe-D-Leu-L-Phe-D-Leu-L-Phe (Boc2; Bachem, Bubendorf Switzerland), was dissolved in dimethyl sulfoxide (DMSO 1%) / sodium chloride (NaCl) 0.9%. 6-keto-prostaglandin $F_{1\alpha}$ ($6kPGF_{1\alpha}$), thromboxane B_2 (TXB_2), prostaglandin E_2 (PGE_2), prostaglandin A_1 (PGA_1), 8-iso prostaglandin A_2 (8-iso PGA_2), prostaglandin E_3 (PGE_3), lipoxin A_4 (LxA_4), lipoxin B_4 (LxB_4), lipoxin A_4 deuterated (LxA_4-d5), resolvin D1 (RvD1), resolvin D2 (RvD2), 7(S)-maresin (7-MaR1), leukotriene B_4 (LTB_4), leukotriene B_5 (LTB_5), leukotriene B_4 deuterated (LTB_4-d4), 10(S),17(S)-protectin (PDx), 18-hydroxyeicosapentaenoic acid (18-HEPE), dihydroxy-eicosatetraenoic acid (5,6-DiHETE), 15-hydroxyeicosatetraenoic acid (15-HETE) and 12-HETE, 8-HETE, 5-HETE, 5-HETE-d8, 17-hydroxy-docosahexaenoic acid (17-HDoHE) and 14-HDoHE, 14,15-epoxyeicosatrienoic acid (14,15-EET) and 11,12-EET, 8,9-EET, 5,6- EET, 5-oxoeicosatetraenoic acid (5-oxo-ETE) were purchased from Cayman

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Chemicals (Ann Arbor, MI, USA). Molecular target nomenclature conformed to BJP's Guide to Receptors and Channels (Alexander *et al.*, 2011).

Study design and surgical procedure

One hundred and twenty mice were randomly assigned to one of 3 isocaloric diets containing 5% fat (w/w) for 9 weeks. A balanced diet providing both n-3 and n-6 fatty acid was used as a control, against two different PUFA-enriched diets, providing either high n-3 or high n-6 intake. Mice were weighed once a week during the diet and the weight gain determined. After 9 weeks, a randomized complete block design has been used to split the experiment into a number of 5 "mini-experiments". The days of the surgery each diet group was split in 4 different experimental groups: (i) sham ischemia, (ii) ischemia (iii) sham ischemia/reperfusion (sham I/R) and (iiii) ischemia/reperfusion (I/R) (n=10 per group). For each surgery day 2 animals of the 3 different diet conditions have been subject to the surgical protocol (24 animals/day for 5 days).

Mice were anaesthetized by an intraperitoneal injection of sodium pentobarbital 50 mg/kg. Animals were kept under anaesthesia during the all period of surgery. The adequacy of anesthesia was monitored with a toe pinch testing to check the animal's reaction. Following abdominal laparotomy, the small bowel was retracted to the left and the superior mesenteric artery (SMA) was temporarily occluded using a microvascular clip (Harvard Apparatus, Les Ulis, France) to cause ischemia. Small bowel and SMA were replaced in mice abdomen and the incision protected with gauze soak with saline solution (NaCl 0.9%). After 50 minutes the clip was gently removed allowing reperfusion. Mice of the ischemia groups were sacrificed immediately after the ischemic period. Mice of I/R groups were sacrificed 5 hours after reperfusion. Sham-operated animals, in which abdominal laparotomy and artery isolation were

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performed without occlusion of the vessel, served as controls and were sacrificed just after the corresponding ischemic (sham ischemia) or I/R (sham I/R) time. After the surgical procedure, two-layer sutures closed the midline incision of the abdominal wall. During the surgical procedure mice were placed on a warming plate (32°C). An independent experiment was performed to determine the effect of Fpr2 endogenous activation during intestinal ischemia reperfusion. Eighty mice were fed with the diet providing high n-6 intake. After 9 weeks, animals were randomized in 4 different sub-groups of 20 animals: sham ischemia, sham I/R, ischemia and I/R as previously described. Ten mice per group were treated by intravenous injection of the tail vein with Boc2 (500 µg/kg). Animals were euthanized at the end of the study for the purpose of sample collection by a lethal overdose of pentobarbital i.p. followed by cervical dislocation

Fatty composition of diets

The three diets were isocaloric and contained 5% fat (w/w) and were designed as previously described (Ducheix *et al.*, 2013). Pellets were prepared by UPAE (INRA, Jouy en Josas). Oils used for experimental diets preparation were grape seed and colza oils (50/50) for the Control diet (Ctrl), grape seed oil (n-6), and colza oil / fish oils (80/20) for a long chain n-3 fatty acid enriched diet (n-3). The EPA enriched fish oil that was used in this study was obtained from Polaris (Quimper, France). The full composition of the diet is given in table 1. The fatty acid composition expressed in percentage of total polyunsaturated fatty acids is provided in table 2. Pellets were stored under vacuum at -20°C. The diets were changed twice a week in each animal cage to avoid oxidative degradation of lipids.

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Assessment of inflammation

Specimens of the ileum were embedded in paraffin. Sections (5 µm) were stained with hematoxylin and eosin. Microscopic histological damage score was performed blinded to the investigator and was based on a semi quantitative scoring system in which the following features were graded: extent of destruction of normal mucosal architecture, presence and degree of cellular infiltration, extent of muscle thickening, presence or absence of crypt abscesses, and presence or absence of goblet cell depletion. The scores for each feature were summed with a maximum possible score of 11 as previously described (Cattaruzza *et al.*, 2006).

Myeloperoxidase (MPO) activity was assessed in the ileum tissue and was used as an index of granulocyte infiltration as previously described (Motta *et al.*, 2012). Chemokine (C-X-C motif) ligand 1 (CXCL1) and chemokine ligand 2 (CCL2) were measured in intestinal tissue by Cytometric Bead Array (BD Biosciences) as previously described (Motta *et al.*, 2012).

Immunohistochemistry

Intestines were cryoprotected, cut into 5 µm sections in a cryostat, and mounted on a Superfrost slide (Thermo Fisher Scientific). Slides were washed in phosphate buffered saline (PBS), 0.5% Triton X-100, and 1% bovine serum albumin (BSA) solution (Sigma) and incubated overnight at 4°C with the primary antibody directed against Fpr2 (1/100, Santa Cruz, Heidelberg, Germany) along with anti-cytokeratin 18 (1/500, Santa Cruz), anti-CD45 (1/500, R&D systems, Lille, France) or anti-Ly-6B.2 (1/400, Bio-Rad AbD Serotec GmbH, Colmar, France). After washes, slides were incubated with appropriate secondary antibody (Alexa Fluor 488 and 555; Invitrogen, Cergy Pontoise, France) for 2 hours. Slides were mounted with Prolong® containing DAPI (Invitrogen). As control procedures, reactions were performed with omission of primary or

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secondary antibodies. Images were acquired using Zeiss LSM-710 confocal microscopes with 63X objective in the inverted configuration (Carl Zeiss MicroImaging Inc., Göttingen, Germany). Images of stained and control slides were collected and processed identically. The quantification of fluorescence was determined using Metamorph software (Molecular Devices).

Real-time PCR analysis

Total RNA from small intestine was isolated with TRIzol reagent (Invitrogen Paisley, UK) and 1µg of the total RNA was then reverse-transcribed with random hexamer oligonucleotides and SuperScript III (Invitrogen), according to the manufacturer's instructions. Amplification was performed with a LightCycler 480 using a SYBR Green I Master kit (Qiagen Ltd, UK) and one of the following mouse primer: *fpr2* (QT00171514, Qiagen), *alox12b* (QT00173551), *alox15* (QT00111034) or *alox5* (QT00258622). Relative expression of the target gene was normalized to expression of the *Hprt* gene, using the $\Delta\Delta C_t$ method (Boue *et al.*, 2011).

Hepatic and intestinal fatty acid profiling

Hepatic fatty acids were analyzed as described previously (Zadravec *et al.*, 2010). Briefly, following homogenization of tissue samples in methanol/5 mM EGTA (2:1, v/v), lipids corresponding to an equivalent of 2 mg of tissue were extracted in the presence of glyceryl triheptadecanoate as an internal standard. The lipid extract in 1ml of heptane was transmethylated with 1 mL of BF3 in methanol (MeOH,1:20, v/v) for 150 min at 100°C, evaporated to dryness, and the FAMEs were extracted with hexane/water (3:1). The organic phase was evaporated to dryness and dissolved in 50 µL ethyl acetate. One microliter of FAME was analyzed by gas-liquid chromatography on a 5890 Hewlett-Packard system (Hewkett-

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Packard, Palo Alto, CA, USA) using a Famewax fused-silica capillary column (30 mm X 0.32 mm i.d., 0.25 µm film thickness; Restek, Belfast, UK). Oven temperature was programmed from 110 to 220°C at a rate of 2°C/min, and the carrier gas was hydrogen (0.5 bar). The injector and the detector were at 225 and 245°C, respectively.

Intestinal lipid extraction

Mouse small intestines were crushed with a FastPrep®-24 Instrument (MP biomedical) in 500 µL of Hank's balanced salt solution (HBSS, Invitrogen) and 5 µL of internal standard mixture (LxA_4-d5, LTB_4-d4 and 5-HETE-d8 at 400 ng/mL in MeOH). After 2 crush cycles (6.5 m/s, 30 s), 10µL is withdrawn for protein quantification and 300 µL of cold methanol was added. Samples were centrifuged at 3000 rpm for 15 min at 4°C. Supernatants were collected, completed to 2 mL in H_2O and submitted to solid-phase extraction using HRX-50 mg 96-well (Macherey Nagel, Hoerd, France). Briefly, after plate conditioning, the sample was loaded at flow rate of 0.1 mL/min. After complete loading, the plate was washed with H_2O/MeOH (90:10, 2 mL) and lipid mediators were eluted with MeOH (2 mL). Solvent was evaporated under nitrogen and samples were dissolved with MeOH and stored at -80 °C for liquid chromatography/tandem mass spectrometry measurements.

Liquid chromatography/tandem mass spectrometry (LC-MS/MS) measurements

By this technique we performed the quantification of $6kPGF_{1\alpha}$, TXB_2, PGE_2, PGA_1, 8-isoPGA_2, PGE_3, LxA_4, LxB_4, RvD1, RvD2, 7-MaR1, LTB_4, LTB_5, PDx, 18-HEPE, 5,6-DiHETE, 15-HETE, 12-HETE, 8-HETE, 5-HETE, 17-HDoHE, 14-HDoHE, 14,15-EET, 11,12-EET, 8,9-EET, 5,6-EET and 5-oxo-ETE in mouse intestinal tissue (Le Faouder *et al.*, 2013). To simultaneously

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separate 27 lipids of interest and 3 deuterated internal standards, LC-MS/MS analysis was performed on ultra high performance liquid chromatography system (UHPLC, Agilent LC1290 Infinity) coupled to Agilent 6460 triple quadrupole MS (Agilent Technologies) equipped with electro-spray ionization operating in negative mode. Reverse-phase UHPLC was performed using ZorBAX SB-C18 column (Agilent Technologies) with a gradient elution. The mobile phases consisted of water, acetonitrile (CAN) and formic acid (FA) (75:25:0.1;v/v/v) (A) and ACN, FA (100:0.1, v/v) (B). The linear gradient was as follows: 0% B at 0 min, 85% B at 8.5 min, 100% B at 9.5 min, 100% B at 10.5 min and 0% B at 12 min. The flow rate was 0.35 mL/min. The autosampler was set at 5°C and the injection volume was 5 µL. Data were acquired in Multiple Reaction Monitoring (MRM) mode with optimized conditions. Peak detection, integration and quantitative analysis were done using Mass Hunter Quantitative analysis software (Agilent Technologies). For each standard, calibration curves were built using 10 solutions at concentration ranging from 0.95 ng/mL to 500 ng/mL. A linear regression with a weight factor of 1/X was applied for each compound. The limit of detection (LOD) and the limit of quantification (LOQ) were determined for the 27 compounds using signal to noise ratio (S/N). The LOD corresponded to the lowest concentration leading to a signal to noise over 3 and LOQ corresponded to the lowest concentration leading to a signal to noise over 10. All values under the LOQ were not considered. Importantly, blank samples were evaluated, and their injection showed no interference (no peak detected), during the analysis. Hierarchical clustering and heat-map were obtained with R (www.r-project.org). PUFA metabolite quantities were transformed to z-scores and clustered based on 1-Pearson correlation coefficient as distance and the Ward algorithm as agglomeration criterion.

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Statistical analysis

Data are presented as mean ± SEM. Analyses were performed using GraphPad Prism 5 software. All data were normally distributed. Between-group comparisons were performed by Student's unpaired 2-tailed t-test. Multiple comparisons within groups I/R were performed by repeated-measures 1-way ANOVA followed by Tukey's procedure. Statistical significance was accepted at $P \leq 0.05$.

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Results:

Effect of dietary intervention on the abundance of fatty acids.

In order to investigate the effect of the dietary intervention on the lipid composition, intestinal and hepatic fatty acids (FA) were profiled by gas chromatography (GC) analysis. Compared to the control group, the ratio between arachidonic (AA) and docosahexaenoic (DHA) acid increased 3-fold in the liver of animals receiving the n-6 diet (Figure 1). Conversely, animals receiving the n-3 diet had a significantly decreased ratio. Even if the total intestinal FA content was the same the dietary intervention had a major impact on the relative FA composition of the gut (Table 3). In the intestine of mice fed with n-6 diet, linoleic acid (LA, C18:2 n-6), C20:2 n-6, arachidonic acid (AA, C20:4 n-6) were significantly increased compared to the control diet group (Table 3). In the intestine of mice fed on n-3 diet, α-linolenic acid (ALA, C18:3 n-3), eicosapentaenoic acid (EPA, C20:5 n-3), C22:5 n-3 and docosahexaenoic acid (DHA, C22:6 n-3) were significantly increased compared to the control diet. On the other hand, in this group C20:2 n-6, C20:3 n-6 and AA were significantly decreased. The ratio between arachidonic acid (AA) and docosahexaenoic acid (DHA) mirrored the changes in the relative abundance of intestinal polyunsaturated fatty acid. During dietary intervention weight gain was weekly assessed and it was found to be similar regardless of diet (Supplementary figure 1)

Long-term n-6 diet prevents intestinal ischemia reperfusion injury

In animals fed the control diet, 50 minutes of ischemia followed by 5 hours of reperfusion resulted in acute inflammation. This was characterized by microscopic damage: fragments of mucosa and red blood cells were found in the lumen. Villi were completely denuded or severely

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damaged, together with loss of crypt architecture. Myeloperoxidase (MPO) activity and chemokine (C-X-C motif) ligand 1 (CXCL1)/ chemokine ligand 2 (CCL2) expressions were also markedly increased in I/R *versus* sham operation in control diet animals (Figure 2 A-E). These inflammatory markers were unchanged in animals receiving a high n-3 diet and then subjected to ischemia-reperfusion. Conversely, the microscopic damage score, MPO activity and CXCL1/CCL2 intestinal expression were significantly decreased in the IR group fed with a high n-6 diet (Figure 2 A-E), compared to control diet group. Taken together these data demonstrate that the administration of a long-term n-6 diet, but not of n-3 diet, protects the gut from the pro-inflammatory damaging consequences of I/R injury.

Effects of diet on the production of intestinal n-6 and n-3 metabolites after ischemia or ischemia followed by reperfusion

We questioned how much the dietary fatty acids might influence the eicosanoid and docosanoid profiles likely to influence I/R induced damage. RvD1, RvD2, 7-MaR1, 14,15-EET, 11,12-EET, 8,9-EET and 5,6-EET were not detectable and PGA_1, 8-isoPGA_2 and 5,6-DiHETE were not quantifiable in intestinal tissue. PUFA metabolites hierarchical clustering was used to reveal the main differences (Figure 3A). The columns represented the average of PUFA metabolite quantity (pg/mg of proteins) of the different group of mice fed Ctrl, n-3 or n-6 diet. PUFA metabolites formed 4 different clusters. The first cluster was composed by product of arachidonic acid metabolism by cyclooxygenase (COX)- (PGE_2, $6kPGF_{1\alpha}$, TXB_2) and lipoxygenase (LOX)-derived metabolites (LTB_4, LxB_4, 5/8/15/12-HETE). The principal characteristic of this cluster was that the quantity of eicosanoids was maximal following ischemia/reperfusion in groups of mice submitted to control diet (Figure 3A). 8/15/12-HETE,

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PGE_2, $6kPGF_{1\alpha}$, TXB_2, LTB_4 and LxB_4 were significantly increased only in control diet group submitted to I/R compared to sham I/R (Supplementary Figure 2). 5-HETE was significantly increased in control diet group submitted to ischemia compared to sham ischemia (Supplementary Figure 2). The second cluster was represented only by LxA_4; its quantity was maximal in group of mice submitted to n-6 diet and to ischemia (Figure 3 A). 5oxo-ETE and $15dPGj_2$ were forming the third cluster characterized by a decrease of these two metabolites by ischemia independently of the diet. The last cluster regrouped all n-3 PUFA metabolites. They were increased in animals submitted to n-3 diet, independently of ischemia or ischemia/reperfusion (Figure 3A).

Due to the protective effect of n-6 diet on ischemia reperfusion injury, we focused our attention on LxA_4. In mice receiving the control diet, the anti-inflammatory mediator LxA_4 was significantly increased compared to sham after ischemia (Figure 3B). In mice submitted to n-6 diet, after ischemia, LxA_4 was significantly increased compared to sham and also compared to mice submitted to control diet and ischemia (Figure 3B). Moreover, in this group of mice we quantified a significant increase in *alox5*, *alox12* and *alox15* mRNA expression compared to mice receiving the control diet after ischemia (Supplementary Figure 3). These findings suggest that diet supplementation is able to change the PUFA metabolites quantity in colonic tissue after ischemia or ischemia/reperfusion by an increase of lipoxygenase expression. Interestingly, after ischemia, the increased LxA_4 in n-6 diet *vs.* control diet suggests a possible protective role for this anti-inflammatory lipid in the protection offered by this diet.

Fpr2 is expressed in mouse small intestine

Since the tissue levels of LxA_4 were modified in some of the groups studied, we also

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investigated the expression of the LxA_4 receptor Fpr2 in the same tissues. Ischemia did not modify *fpr2* expression compared to sham (Figure 4A). We observed an increase of *fpr2* mRNA after I/R in control and n-3 diet groups compared to corresponding sham (Figure 4A). Following I/R, the expression of *fpr2* mRNA was not different in mice that received the n-6 diet, compared to sham (Figure 4A). By immunohistochemistry, we observed that Fpr2 was expressed in the mucosa of murine small intestine (Figure 4B). Fpr2-immunoreactivity co-localized with cytokeratin 18, Ly-6B.2 and partially with CD45-immunoreactivity meaning that Fpr2 was expressed on epithelial cells, neutrophils and on 44% of nucleated hematopoietic cells, respectively (Figure 4B). In those different cell types, no difference of Fpr2 fluorescence intensity was observed (Supplementary Figure 4A) between the different groups or diets. In correlation with MPO activity (Figure 2B), Ly-6B.2 expression was increased following ischemia/reperfusion in control and n-3 diet groups compared to I/R n-6 diet group (Supplementary Figure 4B). Thus, in these two groups, the overexpression of *fpr2* could be the consequence of the infiltration of granulocytes expressing Fpr2. Pictures of immunochemistry control procedures are shown in supplementary figure 4C.

Pharmacological blockade of Fpr2 abrogated the anti-inflammatory effects obtained by the n-6 rich diet.

LxA_4 is an effector of endogenous anti-inflammation acting *via* Fpr2. In mice receiving a high n-6 diet, administration of the Fpr pan antagonist, Boc2 (500 µg/kg before ischemia i.v.) abolished the inhibition of microscopic damage score, MPO activity, CXCL1 and CCL2 expression induced by n-6 diet following I/R (Figure 5A). Administration of Fpr antagonist abrogated also the inhibition of 15-HETE, PGE_2, $6kPGF_{1\alpha}$ and TXB_2 induced by the n-6

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366 enriched diet (Figure 5B). Administration of Boc2 had no effect on the increase of LxA4 induced
367 by ischemia in n-6 diet group (Figure 5C). These findings show that the blockade of the LxA_4
368 receptor (Fpr2) annulled the anti-inflammatory effects obtained by the n-6 rich diet and that n-6
369 supplementation-induced protection after I/R is indeed mediated by FPR2 activation.
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Discussion

In the present study we evaluated the effect of dietary intervention based on different n-6 and n-3 PUFA intake, on injury associated with intestinal I/R. Our data indicate that n-6 PUFA afforded significant protection against I/R-induced tissue injuries, while n-3 PUFA diet had no effect on intestinal damage as determined in these settings. Specifically, in mice subjected to intestinal I/R, n-6 PUFA supplementation in diet decreased mucosal damage, granulocyte recruitment and chemokine expression, compared to animals fed a control balanced diet. Protection against I/R injury was associated with an increase of *alox5, alox12* and *alox15* mRNA expression and consequently of LxA_4 production following ischemia in intestinal tissues from n-6 enriched fatty acid-treated group compared to control diet-fed group. *In vivo* experiments demonstrated that the LxA_4 receptor Fpr2 was involved in the protective effects associated with n-6 PUFA-enriched diet. Collectively, these data make us propose that early during ischemia, an endogenous protective pathway mediated by LxA_4 and its receptor could be triggered by changes in dietary fatty acid.

Our results further confirmed the essential role that AA-derived lipid mediators could play in resolving inflammatory response *in vivo* (Serhan *et al.*, 1988). This notion has been well developed by a number of studies, and AA is now considered not only as a precursor of pro-inflammatory bioactive lipids, but also of potent anti-inflammatory mediators named Lipoxins (Dufton *et al.*, 2010; Morris *et al.*, 2006; Schwab *et al.*, 2006; Wallace, 2006). Indeed, in our experimental conditions, we observed that ischemia and ischemia followed by reperfusion led to an increase of pro-inflammatory bioactive lipids or pathway marker derived from AA metabolism by COX (PGE_2, $6kPGF_{1\alpha}$ TxB_2) and LOX (5-, 8-, 12-, 15-HETE, LTB_4). Simultaneously, potent anti-inflammatory mediators derived from the AA metabolism, LxA_4 and

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LxB_4 were also increased. These two lipoxins are differentially expressed in intestinal tissues over time, LxA_4 being produced from the ischemic period and LxB_4 (his positional isomer) increased only after 5 hours reperfusion. In our study, increased level of LxA_4 was significantly higher in n-6 diet group after ischemia compared to control diet group. However, levels LxB4 (whose receptor(s) is yet to be discovered), were the same in both groups. We have recently reported a mechanism activated by neutrophil/platelet aggregates to increased circulating levels of LxA_4 at the end of 30 min mesenteric ischemia (Brancaleone *et al.*, 2013). This is in agreement with our current data and supports the notion that LxA_4 is an early metabolite of ischemic conditions. The effect of LXA4/Fpr2 system in the protective effects of n-6 diet suggests a minor role for LxB_4 in our experimental conditions.

LxA_4 was identified by Serhan and colleagues in 1984 (Serhan *et al.*, 1984) as a short-lived lipid produced principally by transcellular synthesis (Serhan *et al.*, 1984). LxA_4 is described as a potent endogenous anti-inflammatory bioactive lipid mediator (Serhan *et al.*, 2005). Acting through its receptor, Fpr2 (Lavigne *et al.*, 2002; Perretti *et al.*, 2002; Ye *et al.*, 2009), a G protein-coupled receptor belonging to the formyl peptide receptor family, LxA4 is able to halt leukocyte migration (Chiang *et al.*, 2006; Perretti *et al.*, 2009) and promote macrophage phagocytosis of infectious agents and apoptotic neutrophils (Godson *et al.*, 2000; Maderna *et al.*, 2005). In agreement with our results, several studies have demonstrated a protective role for pharmacologically delivered lipoxins or stable lipoxin analogues in I/R (Bannenberg *et al.*, 2004; Sun *et al.*, 2009; Wu *et al.*, 2012).

Cell-cell interaction is one of the routes by which lipoxins are endogenously biosynthesized by cooperation between 15- and 5-lipoxygenases (LOX) (Serhan *et al.*, 2008).

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Primarily described in granulocytes and later in airway epithelial cells and dendritic cells, metabolism of AA by 15-LOX produces 15S-hydroperoxyeicosatetraenoic acid or the reduced alcohol form 15S-hydroxyeicosatetraenoic acid (15S-HETE) (Levy *et al.*, 1993; Serhan, 1989; Serhan *et al.*, 2008). These metabolites are further transformed into epoxytetraene intermediates by the action of leukocyte 5-LOX yielding LxA_4 and LxB_4 (Levy *et al.*, 1993; Serhan, 1989; Serhan *et al.*, 2008). In the intestine, resident tissue cells such as intestinal epithelial cells expressed 15-LOX and could further contribute to LxA_4 generation by the production of 15S-H(p)ETE (McMahon *et al.*, 2001). Indeed, LxA_4 is synthesized in the human gut by colonic mucosa (Mangino *et al.*, 2006). Interestingly, in pathological conditions such as ulcerative colitis, LxA_4 endogenous production is significantly reduced compared to control patients (Mangino *et al.*, 2006). This decrease was coupled to a lower level of 15-LOX in the mucosa of ulcerative colitis patients, compared to tissues from healthy controls (Mangino *et al.*, 2006). In our experimental conditions, where *alox5* and *alox15* expression and LxA_4 were increased after ischemia before granulocyte infiltration, epithelial cells could be the principal source of 15S-H(p)ETE or 15s-HETE metabolized into LxA_4 by 5-LOX bearing cells (polymorphonuclear leukocytes, macrophages, and mast cells that physiologically populate the gut). LxA_4 acts on a specific target receptor termed formyl-peptide receptor-2/lipoxin A_4 receptor (FPR2/ALX) in humans and Fpr2/3 in mice (Perretti *et al.*, 2002; Ye *et al.*, 2009). FPR2 transduces the pro resolving properties of LXA_4, but also the glucocorticoid regulated protein Annexin A1 and humanin (Dufton *et al.*, 2010). Increasing evidence suggests that members of the FPR family and in particular FPR2 might have an important role in the pathophysiology of I/R injury (Gavins, 2010). In a recent study, the use of *fpr2/3*-deficient mice highlighted the importance of this receptor in I/R; in the absence of Fpr2/3, an aberrant inflammatory response characterized by

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prolonged neutrophil adhesion and infiltration (Brancaleone *et al.*, 2013). Furthermore, exogenous delivery of LxA_4 attenuated I/R-mediated inflammation in wild type but not in Fpr2/3 deficient animals (Brancaleone *et al.*, 2013). In mice receiving a high n-6 diet, administration of the Fpr2 antagonist before ischemia reverted the protective anti-inflammatory effect of this diet. Our data indicate that the protective association between n-6 supplementation and the prevention of post-ischemic damage was due, at least in part, to the interaction between LxA_4 (produced in higher amount in the n-6 group) and its receptor, Fpr2. Human FPR2/ALX was first identified on neutrophils and monocytes (Fiore *et al.*, 1992; Takano *et al.*, 1997). Here, we observed that the murine receptor was expressed also in intestinal epithelial cells, confirming a previous *in vitro* study that had identified Fpr2 in intestinal epithelial cell lines (Goh *et al.*, 2001). Interestingly, *in vitro* studies have identified Fpr2 expression preferentially on the basolateral side of intestinal epithelial cells (Kucharzik *et al.*, 2003). LxA_4 produced on the serosal side of the epithelium by leukocyte-epithelial cell interaction could rapidly signal by activating the receptor on the basolateral side, resulting in potential attenuation of inflammation. LxA_4 promotes anti-inflammatory signals not only by regulating innate immune cells, but also by acting on intestinal epithelial cells. Upon stimulation with bacteria (*Salmonella typhimurium*) (Gewirtz *et al.*, 1998), TNFα (Goh *et al.*, 2001; Gronert *et al.*, 1998) or LPS (Kure *et al.*, 2010), human intestinal epithelial cells release the pro-inflammatory chemokine interleukin-8: this response could be inhibited by cell exposure to LxA_4. In accordance with these data, we observed that after I/R the n-6-enriched diet was associated with a decrease in CXCL1, the murine equivalent of human interleukin-8. Other pro-inflammatory signals, such as the transcription factor NF-κB can be modulated by LxA_4 in epithelial cells (Gewirtz *et al.*, 1998). Such inhibition is also downstream of Fpr2 engagement by LxA_4 (Gewirtz *et al.*, 2002). There is conflicting data regarding how

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much n-6 (i.e. linoleic acid, LA) should be consumed in a healthy diet. Some claim that high LA intake promotes inflammation through accumulation of tissue arachidonic acid (AA) and subsequent production of pro-inflammatory lipid mediators. Human studies indicate that high LA in the diet or circulation is not associated with higher *in vivo* or *ex vivo* pro-inflammatory responses. Several studies have shown that individuals consuming the highest level of LA have the lowest inflammatory status (Fritsche, 2008). In our experimental animal study we did not detect any "pro-inflamamtory phenotype" of the n-6 diet group that underwent a sham operation compared to the n-3 or control diet. Conversely, the n-6 but not the n-3 enriched diet displayed anti-inflammatory properties indicating that the consumption of high n-6 diets may work in specific intestinal scenarios. Exploiting this n-6 dependent endogenous anti-inflammatory loop described here could be of therapeutic impact in diseases in which intestinal ischemia contributes to the pathogenesis, such as neonatal necrotizing enterocolitis (NEC) (Berman *et al.*, 2011). It has been shown that the type and composition of infant diet can regulate the outcome of this pathology. In a clinical study, Carlson et al. found a lower incidence of NEC in infants fed formula containing higher amounts of PUFA compared with controls (Carlson *et al.*, 1998). In the rat NEC model, supplementation of the formula with AA have been shown to reduce the degree of intestinal injury (Lu *et al.*, 2007). Thus in this pathology, delivery of LxA_4, shown to be the most important AA metabolite in our model, could be even more effective than AA-based treatment.

In conclusion these data prompt us to propose that the endogenous anti-inflammatory circuit activated by a selective Fpr2 agonist, LxA_4, could be harnessed by boosting n-6 PUFA diet supplementation as a viable therapeutic approach for the prevention of the ischemia reperfusion injuries

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List of author contributions:

Thomas Gobbetti: acquisition, analysis and interpretation of data; redaction of the manuscript

Simon Ducheix: acquisition and analysis of data

Pauline le Faouder: acquisition and analysis of data; redaction of the manuscript

Jérôme Boué: acquisition and analysis of data

Justine Bertrand-Michel: analysis and interpretation of data

Marc Dubourdeau: interpretation of data; redaction of the manuscript

Hervé Guillou: conception and design; interpretation of data; redaction of the manuscript

Mauro Perretti: conception and design; redaction of the manuscript

Nathalie Vergnolle: conception and design; redaction of the manuscript

Nicolas Cenac: conception and design; acquisition, analysis and interpretation of data; redaction of the manuscript

Thomas Gobbetti and Nicolas Cenac take responsibility for the integrity of the work as a whole, from inception to published article

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699 **Table 1: Composition in % of the 3 isocaloric diets**

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	Control	n-6	n-3
Cellulose	2	2	2
Casein	22	22	22
Starch	43.6	43.6	43.6
Methionine	0.2	0.2	0.2
Sucrose	21.8	21.8	21.8
Minerals	4.5	4.5	4.5
Vitamins	1.0	1.0	1.0
Grape Seed Oil	2.5	5	0
Colza Oil	2.5	0	4
Fish Oil	0	0	1

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702 **Table 2: Composition in % of polyunsaturated fatty acid of the 3 isocaloric diets containing**

703 **5% fat (w/w)**

	Control	n-6	n-3
C12:0	0.0	0.0	0.0
C14:0	0.0	0.0	0.0
C16:0	6.0	6.5	4.5
C18:0	2.5	4.2	0.7
C18:1n-9	38.8	18.4	47.4
C18:2n-6	47.4	70.0	19.9
C18:3n-3	5.2	1.0	7.6
C20:5n-3	0.0	0.0	17.0
C22:6n-3	0.0	0.0	3.0
n-6/n-3 ratio	10	70	0.72

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Table 3: Concentration of fatty acid (nmol/mg of protein) in mouse intestine. Mice were submitted to 9 weeks of control, n-3 or n-6 diet before fatty acid (FA) metabolite extraction. Data are express as mean ± SEM, n=10. *p<0.05 vs. control diet group. SAFA: saturated fatty acid; MUFA: monounsaturated fatty acid; PUFA: polyunsaturated fatty acid; LA: linoleic acid; ALA: α-linolenic acid; AA: arachidonic acid; EPA: eicosapentaenoic acid; DHA: docosahexaenoic acid.

	Diet		
	Control	n-3	n-6
10:0	0.00	0.00	0.00
12:0	0.00	0.00	0.00
14:0	0.00	5.39 ± 2.57	1.02 ± 1.02
15:0	0.00	0.00	0.00
16:0	286.85 ± 43.95	349.08 ± 75.12	329 ± 79.41
18:0	185.97 ± 23.68	147.24 ± 21.21	127.53 ± 42.45
20:0	0.00	0.00	0.00
21:0	0.00	0.00	0.00
22:0	0.00	0.00	0.00
14:1 n-5	0.00	0.00	0.00
15:1 n-5	0.00	0.00	0.00
16:1 n-9	0.00	0.00	0.00
16:1 n-7	150.83 ± 13.09	157.22 ± 38.09	146.05 ± 14.37
17:1 n-7	0.00	0.00	0.00
18:1 n-9	413.22 ± 68.93	450.11 ± 49.71	445.18 ± 65.70
18:1 n-7	100.73 ± 17.17	93.00 ± 11.58	94.35 ± 19.73
20:1 n-9	0.00	0.00	0.00
22:1 n-9	0.00	0.00	0.00
24:1 n-9	0.00	0.00	0.00
18:2 n-6 (LA)	84.56 ± 18.38	69.76 ± 10.07	209.05 ± 23.64 *
18:3 n-6	0.00	0.00	0.00
18:3 n-3 (ALA)	0.00	15.65 ± 1.65 *	0.00
20:2 n-6	3.41 ± 1.50	0.00 *	6.97 ± 0.92 *
20:3 n-3	0.00	0.00	0.00
20:3 n-6	18.29 ± 1.03	8.56 ± 0.86 *	13.47 ± 1.34
20:4 n-6 (AA)	39.95 ± 2.34	13.81 ± 1.19 *	60.37 ± 5.70 *
20:5 n-3 (EPA)	0.00	10.78 ± 1.86 *	0.00
22:5 n-3	0.00	5.69 ± 0.78 *	0.00
22:2 n-6	0.00	0.00	0.00
22:4 n-6	0.00	0.00	0.00
22:5 n-6	0.00	0.00	0.00
22:6 n-3 (DHA)	1.88 ± 1.21	19.87 ± 3.14 *	0.93 ± 0.52
20:4 n-6 / 22:6 n-3	9.51 ± 0.75	0.73 ± 0.07 *	44.09 ± 11.34 *
Total SAFA	472.82 ± 67.64	501.71 ± 98.91	457.98 ± 122.88
Total MUFA	664.78 ± 99.19	700.33 ± 99.38	685.58 ± 99.80
Total PUFA	148.08 ± 24.46	144.50 ± 19.93	290.78 ± 32.30 *
Total FA	1284 ± 151	1345 ± 162	1432 ± 114

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Figure legends

Figure 1: Effect of dietary intervention on the hepatic fatty acids composition. Total arachidonic acid (C20:4 n-6) and docosahexaenoic acid (C22:6 n-3) ratio in liver of mice submitted to control, n-3 or n-6 PUFA diet for 9 weeks. Values are mean ± SEM, n= 6-8 per group. ** $p<0.01$, *** $p<0.001$, significantly different from control diet group.

Figure 2: Inflammation of the intestine induced by I/R. (**A**) representative hematoxylin and eosin-stained histological sections of small intestine from sham-operated mouse or mouse submitted to I/R and receiving control, n-3 or n-6 PUFA diet. Different inflammatory parameters were followed: microscopic damage scores (**B**), myeloperoxidase activity (MPO) (**C**) in sham (white bar) or I/R (black bar) mice exposed to control, n-3 or n-6 PUFA diet for 9 weeks. (**D-E**) CXCL1 and CCL2 expressions were quantified in I/R mice *versus* corresponding sham, exposed to control (white bar), n-3 (black bar) or n-6 (gray bar) PUFA diet. Values are mean ± SEM, n= 6-8 per group. * $p<0.05$, ** $p<0.01$, *** $p<0.001$, significantly different from sham; + $p<0.05$, ++ $p<0.01$, significantly different from control diet group.

Figure 3: Quantification of PUFA metabolites in intestine of mice exposed to I/R and to the three different diets. (A) Heat-map and clustering of quantified PUFA metabolites by liquid chromatography-tandem mass spectrometry. The heatmap represents a color coding of average z-scores obtained for the different PUFA metabolites (in row) and the groups of mice (in column). A red color indicates a quantity greater than the mean of the row, a green color indicates a quantity lower than the mean and black color a quantity close to the mean. The dendrogram reflects the correlation of the PUFA metabolites. The dotted red line represents the clusters. (**B**) Synthesis of lipoxin A_4 (LxA_4) was measured by liquid chromatography-tandem mass spectrometry in sham ischemia (white bar), sham I/R animals (stripped bar) and following 50

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minutes of ischemia (gray bar) or 50 minutes of ischemia and 5 hours of reperfusion (I/R, black bar). Data are expressed in pg/mg protein and represent means ± SEM of 6 to 8 animals per group. ** p<0.01, *** p<0.001, significantly different from corresponding sham; ++ p<0.01, significantly different from ischemia control diet group

Figure 4: Expression of FPR2 in mouse small intestine. (**A**) FPR2 mRNA expression was quantified in I/R mice *versus* corresponding sham, exposed to control (white bar), n-3 (black bar) or n-6 (gray bar) PUFA diet. (**B**) Pictures of FPR2, cytokeratin 18, CD45 and Ly-6B.2 immunoreactivity detected in the small intestine of mice exposed to sham and to n-6 PUFA diet (scale: 50 µm). Values are mean ± SEM, n= 6-8 per group. * p <0.05, ** p<0.01, significantly different from corresponding ischemia group.

Figure 5: Role of FPR2 antagonist in n-6 PUFA diet anti-inflammatory effects. (A) Different inflammatory parameters were followed: myeloperoxidase activity, microscopic damage scores, chemokine (C-X-C motif) ligand 1 (CXCL1) expression and chemokine ligand 2 (CCL2) expression, in sham (grey bar) or I/R (black bar) mice treated with FPR2 antagonist (BOC-2, 500 µg/kg before ischemia i.v.) and exposed to n-6 PUFA diet for 9 weeks. (**B**) N-6 PUFA derived metabolites in sham (grey bar) or I/R (black bar) mice treated with FPR2 antagonist (BOC-2, 500 µg/kg before ischemia i.v.) and exposed to n-6 PUFA diet for 9 weeks. Data are expressed as percentage of inhibition of I/R induced effect in control diet group. Values are mean ± SEM, n= 6-8 per group. * p <0.05, ** p<0.01, *** p<0.001, significantly different from sham; + p <0.05, ++ p<0.01, significantly different from I/R vehicle group.

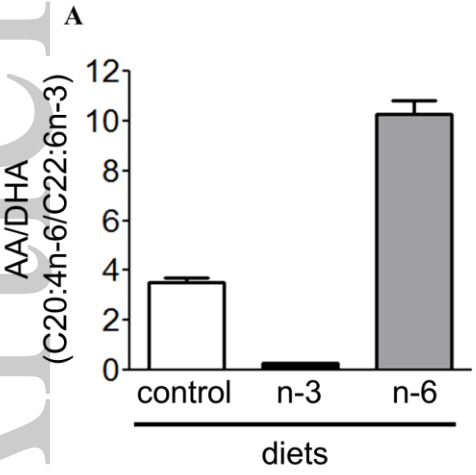
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Figure 1



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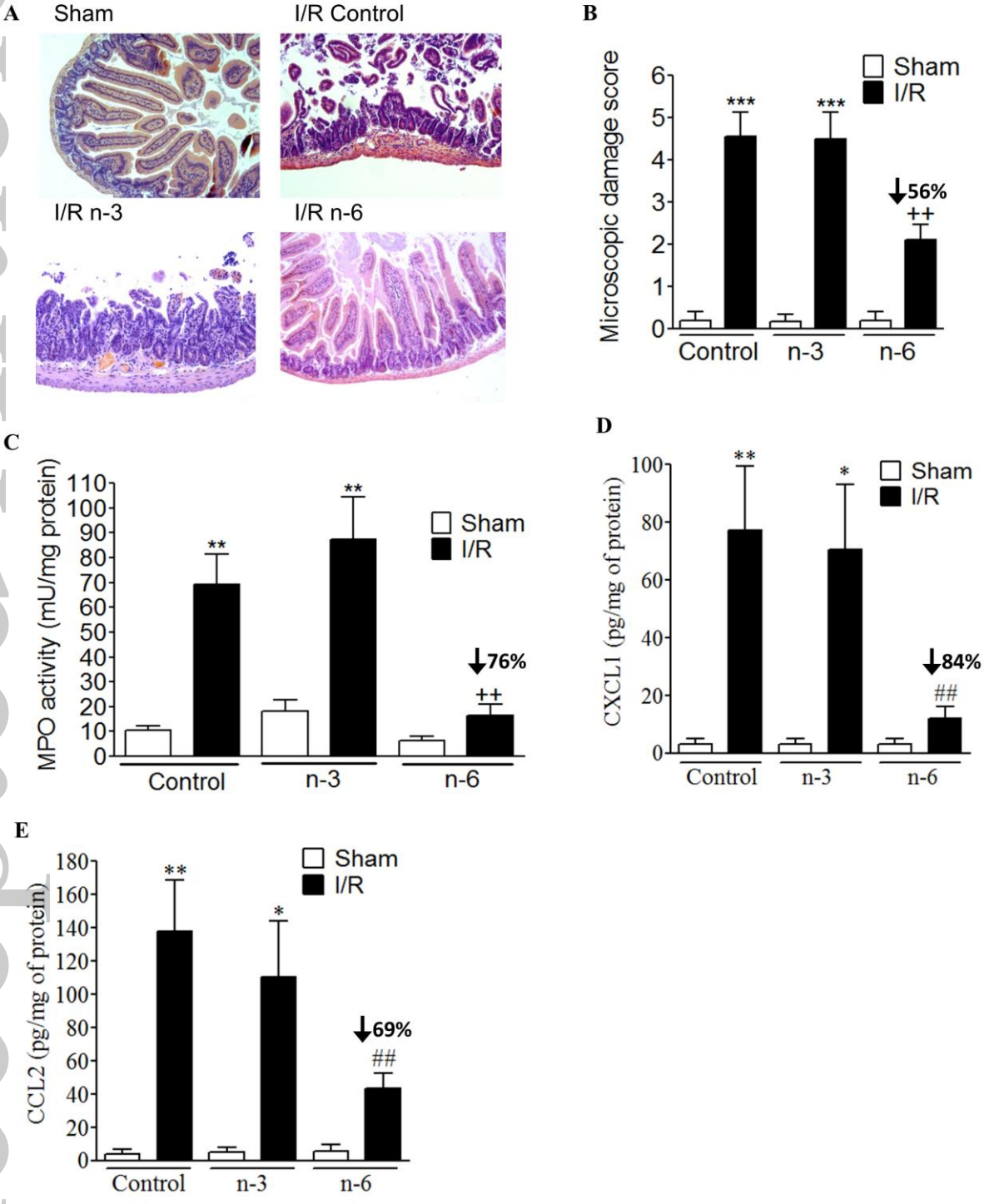
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Figure 2



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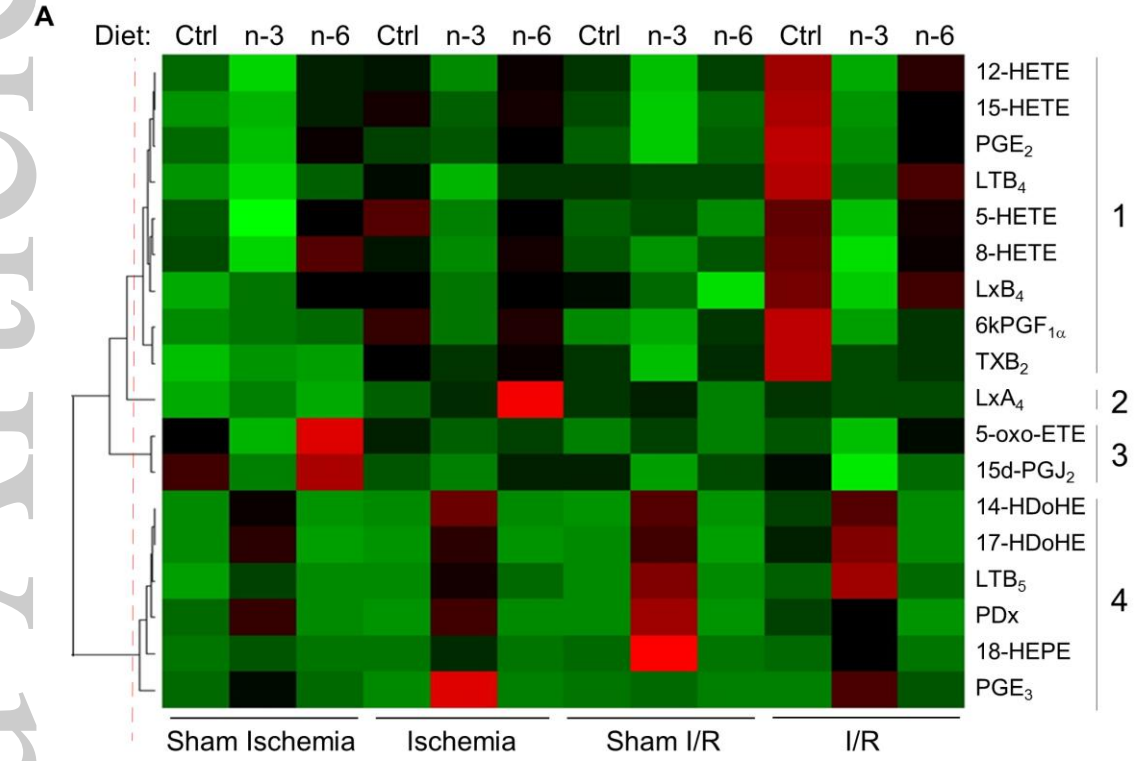
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Figure 3



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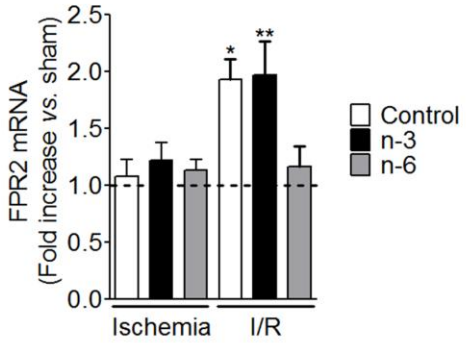
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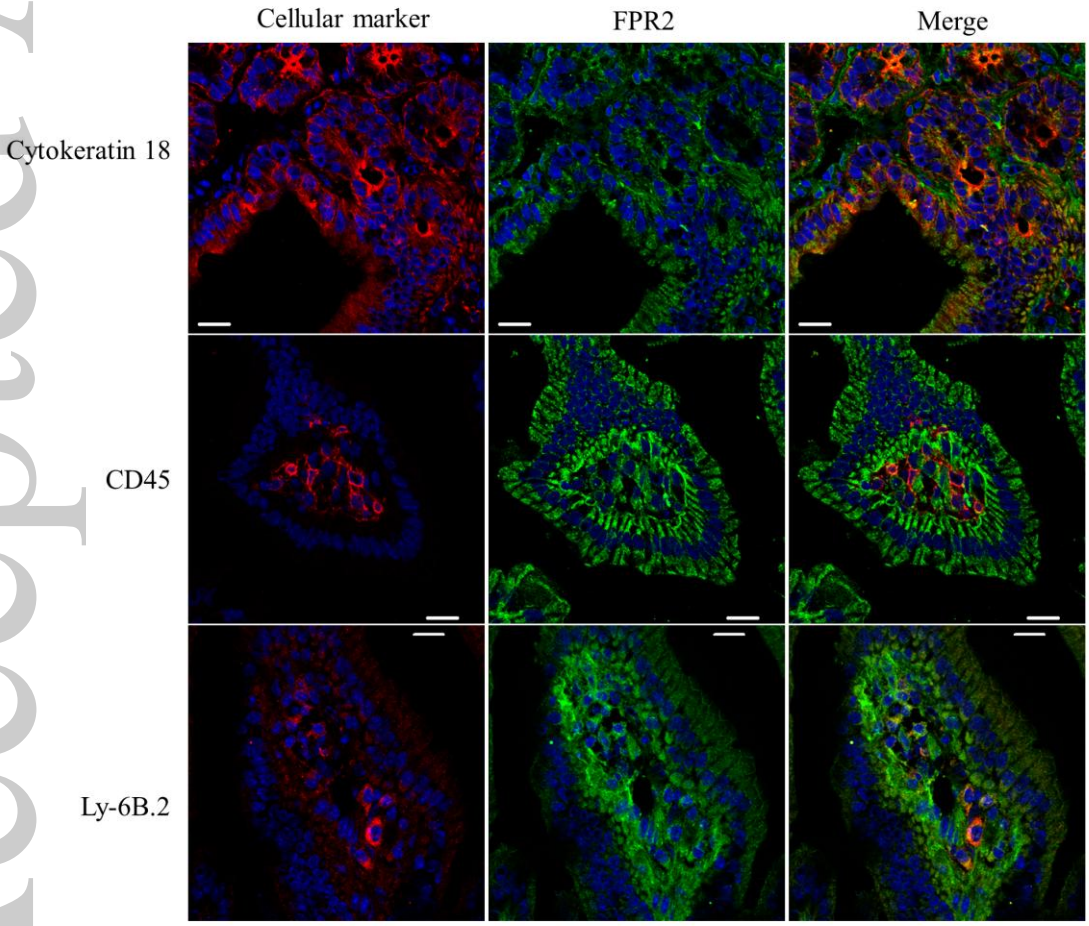
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Figure 4





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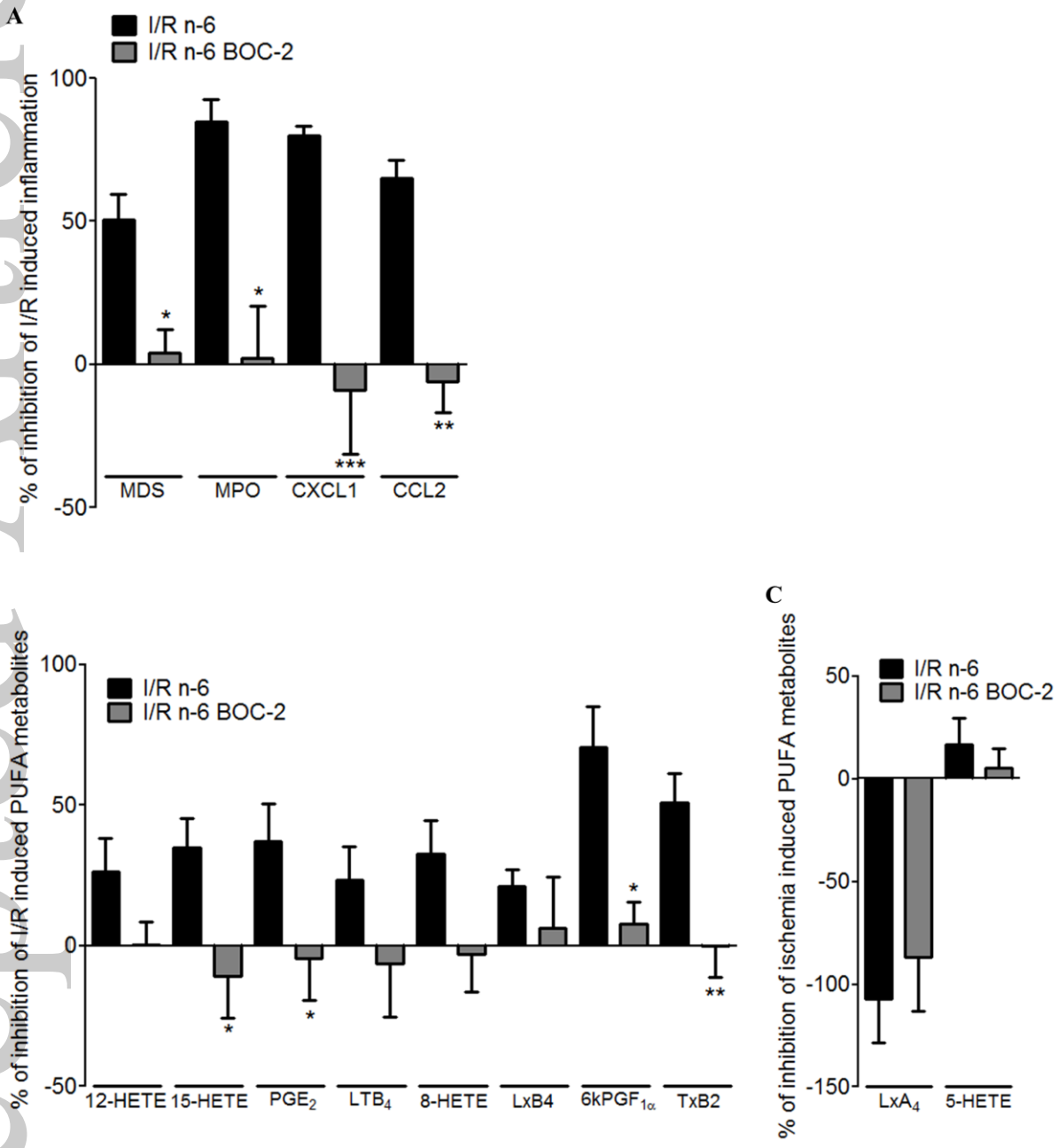
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Figure 5



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