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## **Dietary omega-3 PUFA improved tubular function after ischemia induced acute kidney injury in mice but did not attenuate impairment of renal function**

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1 **Dietary omega-3 PUFA improved tubular function after ischemia induced acute kidney**  
2 **injury in mice but did not attenuate impairment of renal function**

3  
4 Katharina Rund<sup>1#</sup>, Shu Peng<sup>2#</sup>, Robert Greite<sup>2</sup>, Cornelius Claaßen<sup>1</sup>, Fabian Nolte<sup>1</sup>, Camille  
5 Oger<sup>3</sup>, Jean-Marie Galano<sup>3</sup>, Laurence Balas<sup>3</sup>, Thierry Durand<sup>3</sup>, Rongjun Chen<sup>2</sup>, Faikah  
6 Gueler<sup>2\*#</sup>, Nils Helge Schebb<sup>1\*#</sup>

7 <sup>1</sup> Chair of Food Chemistry, Faculty of Mathematics and Natural Sciences, University of  
8 Wuppertal, Wuppertal, Germany

9 <sup>2</sup> Nephrology, Hannover Medical School, Hannover, Germany

10 <sup>3</sup> Institut des Biomolécules Max Mousseron (IBMM), UMR 5247 CNRS, Université de  
11 Montpellier, ENSCM, France

12  
13  
14 # Authors contributed equally

15  
16 \*Corresponding author (Tel: +49 202-439-3457; E-mail: [nils@schebb-web.de](mailto:nils@schebb-web.de), postal address:  
17 Chair of Food Chemistry, Faculty of Mathematics and Natural Sciences, University of  
18 Wuppertal, Gaußstr. 20, 42119 Wuppertal)

20 **Abstract**

21 Background: Acute kidney injury (AKI) is an important complication after major surgery and solid  
22 organ transplantation. Here, we present a dietary omega-3 polyunsaturated fatty acid (n3-PUFA)  
23 supplementation study to investigate whether pre-treatment can reduce ischemia induced AKI in  
24 mice.

25 Methods: Male 12-14 week old C57BL/6J mice received a linoleic acid rich sunflower oil based  
26 standard diet containing 10% fat (STD) or the same diet enriched with n3-PUFA (containing  
27 1% EPA and 1% DHA) (STD+n3). After 14 days of feeding bilateral 30 min renal ischemia  
28 reperfusion injury (IRI) was conducted to induce AKI and mice were sacrificed at 24 h. Serum-  
29 creatinine and blood urea nitrogen (BUN) as well as liver enzyme elevation were measured.  
30 Kidney damage was analyzed by histology and immunohistochemistry. Furthermore,  
31 pro-inflammatory cytokines (IL-6, MCP-1) were determined by qPCR. FA and oxylipin pattern  
32 were quantified in blood and kidneys by GC-FID and LC-MS/MS, respectively.

33 Results: n3-PUFA supplementation prior to renal IRI increased systemic and renal levels of  
34 n3-PUFA. Consistently, eicosanoids and other oxylipins derived from n3-PUFA including  
35 precursors of specialized pro-resolving mediators were elevated while n6-PUFA derived  
36 mediators such as pro-inflammatory prostaglandins were decreased. Feeding of n3-PUFA did  
37 not attenuate renal function impairment, morphological renal damage and inflammation  
38 characterized by IL-6 and MCP-1 elevation or neutrophil infiltration. However, the tubular  
39 transport marker alpha-1 microglobulin (A1M) was significantly higher expressed in proximal  
40 tubular epithelial cells of STD+n3 compared to STD fed mice. This indicates a better integrity of  
41 proximal tubular epithelial cells and thus significant protection of tubular function. In addition,  
42 heme oxygenase-1 (HO-1) which protects tubular function was also up-regulated in the  
43 treatment group receiving n3-PUFA supplemented chow.

44 Discussion: We showed that n3-PUFA pre-treatment did not affect overall renal function or renal  
45 inflammation in a mouse model of moderate ischemia induced AKI, but tubular transport was

46 improved. In conclusion, n3-PUFA food supplementation altered the oxylipin levels significantly  
47 but did not protect from renal function deterioration or attenuate ischemia induced renal  
48 inflammation.

49

50 **Introduction**

51 Acute kidney injury (AKI) is a frequent complication after major cardiac surgery, solid organ  
52 transplantation and also after trauma surgery. Onset of AKI increases morbidity and mortality of  
53 the patients and is characterized by an increase in serum creatinine and/or reduction of the  
54 glomerular filtration rate (GFR) [1]. In a review of 25,182 trauma patients post-traumatic AKI was  
55 as high as 24% and these patients had a 3.4-fold higher risk of death compared to non-AKI  
56 patients [2]. Also, after myocardial infarction mortality was 3-fold increased in patients with AKI  
57 and incidence of major cardiac events (MACE) was with 26% higher than in non-AKI patients  
58 during the three year follow-up [3]. It has been shown that AKI is not only an acute event but  
59 also increases the risk for chronic kidney disease (CKD) [4]. Even if the serum creatinine  
60 elevation returns to normal levels shortly after AKI a significant proportion of patients will develop  
61 CKD or will even proceed to end stage renal disease with the need of renal replacement therapy  
62 [5]

63 Renal ischemia reperfusion (IRI) injury due to hypotension, major bleeding and hypoxia causes  
64 intrarenal vasoconstriction with activation of pro-oxidative mechanisms, release of pro-  
65 inflammatory cytokines and subsequent leukocyte invasion [6-9]. The severity of AKI correlates  
66 with the long-term outcome and progression to CKD [5].

67 Established strategies to prevent or attenuate AKI even in scheduled surgeries such as cardiac  
68 valve replacement are lacking. Several studies discussed a beneficial role of omega-3  
69 polyunsaturated fatty acids (n3-PUFA) in the context of kidney diseases [10-19]. For the  
70 prevention of cardiovascular diseases the nutritional-status of n3-PUFA is a well-established risk  
71 factor [20, 21]. The higher the incorporation of n3-PUFA, determined by the relative content of  
72 EPA and DHA in the cells, typically red blood cells [20, 22] the lower the risk for cardiovascular  
73 diseases and mortality [23]. It has to be noted that n3-PUFA are essential constituents of the  
74 human diet and that the nutritional status and not the intake of a n3-PUFA supplement is related

75 to their physiological effects. Thus, if only the intake of n3-PUFA by supplementation is  
76 correlated to the risk for cardiovascular diseases the outcome is less clear [24, 25].  
77 In the context of renal diseases effects of n3-PUFA supplementation on the outcome in kidney  
78 transplantation are inconsistent [26]. However, in some experimental AKI models the  
79 administration of n3-PUFA correlated with an improved kidney function [10-15].  
80 The molecular mechanisms underlying beneficial effects of n3-PUFA include direct actions, e.g.  
81 binding to ion channels or transcription factors, e.g. NFκB or PPARγ, thereby reducing the  
82 expression of pro-inflammatory and activating the expression of anti-inflammatory genes,  
83 respectively. As constituents of membranes, e.g. predominantly in phospholipids, n3-PUFA also  
84 impact membrane structure and fluidity [27]. However, it is beyond doubt that a relevant part of  
85 the physiological effects of n3-PUFA are mediated by their oxygenated products, i.e.  
86 eicosanoids and other oxylipins [27-29]. In the so-called arachidonic acid (ARA) cascade PUFA  
87 serve as substrates for enzymatic and non-enzymatic conversion resulting in a multitude of lipid  
88 mediators from both n6- and n3-PUFA (Fig. 1) [27, 28, 30]. Enzymatic conversion of PUFA in the  
89 ARA cascade comprises three major pathways: (I) cyclooxygenases (COX) lead to the formation  
90 of prostanoids and thromboxanes, (II) cytochrome P450 monooxygenases (CYP) give rise to  
91 epoxy-PUFA and terminal hydroxy-PUFA and (III) lipoxygenases lead via hydroperoxy-PUFA to  
92 hydroxy-PUFA and leukotrienes [31]. Structurally similar to these enzymatically formed products  
93 oxidized PUFA also arise during non-enzymatic autoxidation, e.g. isoprostanes, which also have  
94 been shown to possess biological activity [32, 33]. In the kidney, oxylipins have important  
95 functions in (patho-)physiology, e.g. regulation of renal blood flow, glomerular filtration rate and  
96 tubular transport function [34-37]. As n3-PUFA compete with ARA for conversion dietary n3-  
97 PUFA supplementation impacts the overall oxylipin profile resulting in a shift from predominantly  
98 pro-inflammatory, e.g. 2-series prostaglandins, towards n3-PUFA derived oxylipins with less  
99 potency or even anti-inflammatory properties [27]. Furthermore, in recent years activity of LOX  
100 with n3-PUFA have been related to the formation of multiple hydroxylated PUFA, e.g. resolvins,

101 maresins and protectins which have been attributed to be actively involved in the resolution of  
102 inflammation [38], though several studies fail to detect them in biological samples [39, 40].  
103 Changes in the oxylipin pattern in the context of kidney function in AKI have been sparsely  
104 investigated. Therefore, we used a well characterized model of ischemia induced AKI in mice  
105 [41, 42] to study the effect of dietary n3-PUFA (EPA and DHA) supplementation based on a  
106 linoleic acid rich western-like diet [43] for 14 days prior to surgery on the fatty acid composition  
107 and oxylipin pattern in blood and tissue in comparison to renal damage and inflammation at 24 h  
108 after injury.  
109

110 **Materials and methods**

111 Chemicals

112 Oxylin and deuterated oxylin standards were purchased from Cayman Chemicals (local  
113 distributor: Biomol, Hamburg, Germany). Methyl pentacosanoate (FAME C25:0) was obtained  
114 from Santa Cruz Biotechnology (Heidelberg, Germany). PD1 and isoprostane standards were  
115 included in the method based on in-house synthesis as described [40, 44]. HPLC grade and LC-  
116 MS-grade methanol (MeOH), LC-MS-grade acetonitrile (ACN), LC-MS-grade isopropanol, LC-  
117 MS-grade acetic acid and HPLC grade methyl *tert*-butyl ether (Acros Organics) were purchased  
118 from Fisher Scientific (Schwerte, Germany). Sodium hydrogen phosphate and *n*-hexane (HPLC  
119 grade) were obtained from Carl Roth (Karlsruhe, Germany). Ammonium acetate (p.a.) was  
120 obtained from Merck (Darmstadt, Germany) and potassium hydroxide (85%) from Gruessing  
121 GmbH (Filsum, Germany). Ethyl acetate, acetyl chloride and potassium carbonate (anhydrous)  
122 were purchased from Sigma Aldrich (Schnelldorf, Germany).

123

124 Feeding experiment and renal ischemia reperfusion injury in mice

125 C57BL/6J<sup>ham-ztm</sup> male mice (12–14 weeks of age) were obtained from the institute of laboratory  
126 animal science (Hannover Medical School, Germany). Mice were cared for in accordance with  
127 the institution's guidelines for experimental animal welfare and with the guidelines of the  
128 American Physiological Society. All experiments were approved by the animal protection  
129 committee of the local authorities (Lower Saxony state department for food safety and animal  
130 welfare, LAVES; approval 33.19-42502-04-14/1657). Mice were housed under conventional  
131 conditions with a 14/10 h light/dark cycle. Mice were divided into two feeding groups receiving  
132 either a sunflower oil based standard diet (STD) or the same diet enriched with n3-PUFA  
133 containing 1% eicosapentaenoic acid (EPA) and 1% docosahexaenoic acid (DHA) as ethyl  
134 esters (10% each in fat; STD+n3) based on a standard experimental diet (ssniff Spezialdiaeten  
135 GmbH, Soest, Germany). Both diets contained in total 10% fat. During the whole feeding period

136 every 2-3 days fresh chow was provided and animals had free access to the food and domestic  
137 quality drinking water.

138 After 14 days of feeding, renal IRI was initiated in general isoflurane anesthesia (5% induction,  
139 2% maintenance) combined with iv butorphanol as analgetic treatment. IRI was induced by  
140 transient bilateral renal pedicle clamping for 30 min using a non-traumatic vascular clamp [45].  
141 Mice were sacrificed 24 h after reperfusion by deep general anesthesia and total body perfusion  
142 with ice cold PBS [45].

143 IRI experiments were conducted in three sets each with n=7-8 animals in the STD and STD+n3  
144 fed group. In one of these experiments additionally a sham group (n=5) receiving STD chow was  
145 included which underwent midline laparotomy but without renal pedicle clamping.

146 Blood was collected at baseline (before starting the feeding) and at 24 h after reperfusion. An  
147 aliquot of 30  $\mu$ L whole blood was diluted with 150  $\mu$ L deionized water for fatty acid analysis and  
148 EDTA-plasma was generated by centrifugation (4000  $\times$  g, 10 min, 4°C) and stored at -80°C until  
149 analysis for clinical chemistry and oxylipins. Kidneys were collected at endpoint, dissected and  
150 immediately processed as follows: one piece was fixed in paraformaldehyde for histology, one  
151 piece was stored in RNAlater for qPCR and one piece was shock frozen in liquid nitrogen and  
152 stored at -80°C for fatty acid and oxylipin analysis. Renal function (serum-creatinine and BUN)  
153 and liver enzymes (aspartate transaminase: AST, alanine aminotransferase: ALT) were  
154 measured in EDTA-plasma by an Olympus analyzer (AU400) in an automated fashion according  
155 to the manufacturer's instruction.

156

#### 157 Histology and immunohistochemistry

158 The middle part of the kidney was fixed in 4% paraformaldehyde (PFA) overnight and embedded  
159 in paraffin. Two  $\mu$ m paraffin sections were cut and PAS (Perjodic Schiff's Acid) stain according  
160 to standard diagnostic protocols was done. For determination of AKI scores a semi-quantitative  
161 grading system was used: 0 = focal AKI with <5% of tubuli of the cortex affected, 1 = mild AKI

162 with 5-25% of tubuli affected, 2 = moderate AKI with 26-50% of tubuli affected, 3 = severe AKI  
163 with 51-75% of the tubuli affected, 4 = very severe AKI with > 75% of tubuli affected.  
164 Immunohistochemistry for neutrophil infiltration (Gr-1 antibody, Biorad), the tubular function  
165 marker alpha-1 microglobulin (A1M; gift from Magnus Gram, Lund University) and heme  
166 oxygenase-1 (HO-1 antibody; Enzo Life Sciences, Switzerland) was done on paraffin sections.  
167 Sections were incubated with trypsin for 15 min at 37°C for antigen retrieval. Nonspecific binding  
168 sites were blocked with 10% normal donkey serum (Jackson ImmunoResearch Lab, West  
169 Grove, USA) for 30 min and then primary antibodies (Invitrogen, California, USA) were  
170 incubated for 60 min at room temperature in the dark. Afterwards secondary antibodies  
171 (Invitrogen, California, USA) were incubated for additional 60 min in the dark. Analysis was  
172 performed in a blinded manner using a Leica imaging microscope. Gr-1 positive neutrophil  
173 infiltration was scored with a 0-4 grading system in 10 different view fields (VF) per section in  
174 200-fold magnification: 0 = no infiltrates, 1 = mild infiltrates with <5 cells/VF, 2 = moderate  
175 infiltrates with 6-10 cells/VF, 3 = severe infiltration with 11-25 cells/VF, 4 = very severe infiltration  
176 with >25 cells/VF. Semiquantitative analysis of the percentage of A1M or HO-1 positive proximal  
177 tubuli in the cortex was done in 10 different VF per section.

178

179 Detection of pro-inflammatory cytokines by qPCR

180 For mRNA work-up one part of the kidneys was fixed in RNAlater immediately. For total RNA  
181 extraction the RNeasy mini kit system (Qiagen, Hilden, Germany) was used and RNA was  
182 transcribed with Qiagen mini kits. For quantitative PCR (qPCR) 1 µg of DNase-treated total RNA  
183 was reverse transcribed using Superscript II reverse transcriptase (Invitrogen) and qPCR was  
184 performed on a Lightcycler 420 II (Roche Diagnostics, Penzberg, Germany) using FastStart  
185 Sybr-Green chemistry. Gene-specific primers for MCP-1 (QT00167832) and IL-6 (QT00098875)  
186 were used. For normalization HPRT (QT00166768) was used as housekeeping gene.  
187 Quantification was carried out using qgene software.

188  
189 Extraction and quantification of fatty acids  
190 The fatty acid composition was analyzed in diluted whole blood (15  $\mu$ L blood + 75  $\mu$ L water) and  
191 kidney tissue (15  $\pm$  2 mg) as fatty acid methyl esters using gas chromatography with flame  
192 ionization detection (GC-FID) as described with slight modifications [46]. Briefly, 10  $\mu$ L internal  
193 standard (FAME C25:0, 750  $\mu$ M) was added to diluted whole blood and kidney tissue samples.  
194 Kidney tissue was homogenized in 300  $\mu$ L MeOH and 50  $\mu$ L water using two stainless steel  
195 beads (3 mm, 5 min, 25 Hz) with a vibration ball mill (MM 400, Retsch, Haan, Germany). Lipids  
196 were extracted with methanol/methyl *tert*-butyl ether and the lipid extract was trans-esterified to  
197 fatty acid methyl esters using methanolic hydrogen chloride. The calculation of the absolute fatty  
198 acid concentrations and relative pattern was based on response factors as described [46].

199  
200 Extraction and quantification of oxylipins  
201 Oxylipins in plasma and kidney tissue were extracted using anion exchange Bond Elut Certify II  
202 SPE cartridges (Agilent, Waldbronn, Germany) as described with modifications [40, 44]. In the  
203 first step of oxylipin extraction 10  $\mu$ L antioxidant solution and internal standards were added to  
204 each sample.

205 For the determination of free oxylipins in plasma (150-200  $\mu$ L) the 2.8-fold volume of MeOH was  
206 added and the samples were stored at -80°C for 30 min. After centrifugation the supernatant  
207 was diluted with disodium hydrogen phosphate buffer yielding a MeOH content  $\leq$ 18% (pH 6.0)  
208 and loaded on the preconditioned SPE cartridge.

209 For analysis of free oxylipins in kidney tissue (50  $\pm$  5 mg) samples were homogenized with  
210 300  $\mu$ L MeOH as described [47]. After centrifugation the supernatant was diluted with 2.7 mL  
211 sodium phosphate buffer (pH 6.0) and loaded on the preconditioned SPE cartridge.

212 For quantification of total, i.e. free and esterified oxylipins in kidney tissue (20  $\pm$  2 mg) samples  
213 were homogenized in 400  $\mu$ L isopropanol and stored for 30 min at -80°C. Samples were

214 centrifuged and the supernatant was hydrolyzed (300  $\mu$ L 1.5M KOH (75/25, MeOH/water, v/v),  
215 immediately neutralized with acetic acid, diluted with 2 mL sodium phosphate buffer (pH = 5.5)  
216 and loaded on the preconditioned SPE cartridge.

217 The SPE procedure was carried out as described and samples were analyzed by LC-MS/MS  
218 (QTRAP, Sciex, Darmstadt, Germany) in scheduled selected reaction monitoring mode following  
219 negative electrospray ionization as described [40, 44].

220

#### 221 Data analysis

222 Data evaluation and statistical analyses were performed using GraphPad Prism software for  
223 Windows (version 5.0, La Jolla California USA). Data are presented as mean  $\pm$  standard error of  
224 mean (SEM). For oxylipin and fatty acid analysis for the calculation of the mean  $\frac{1}{2}$  lower limit of  
225 quantification (LLOQ) was used if the concentration was below the LLOQ. The concentration  
226 was set to LLOQ if the analyte could not be quantified in more than 50% of the samples in one  
227 group. For comparison of two groups two-tailed unpaired student's t-test was used. For multiple  
228 comparisons ANOVA with post hoc Tukey correction was applied. Differences were considered  
229 significant at a p-value  $<0.05$  (\* $p<0.05$ , \*\* $p<0.01$ , \*\*\* $p<0.001$ ).

230

231 **Results**

232 Bodyweight and food intake of both feeding groups were not changed between the STD and the  
233 STD+n3 diet.

234 **Blood and tissue pattern of fatty acids and their oxidative metabolites**

235 After 14 days of feeding an n3-PUFA enriched sunflower oil based diet (1% EPA and 1% DHA in  
236 the chow, STD+n3) absolute and relative levels of n3-PUFA in the kidney and in the circulation  
237 were significantly increased predominantly at the expense of n6-PUFA compared to mice on the  
238 STD diet (Fig. 2 A+C). Considering the very low levels of EPA in tissue and in the circulation of  
239 mice on the STD diet, especially relative levels of EPA were massively increased in response to  
240 the STD+n3 diet. Interestingly, in whole blood the STD+n3 diet led to an overall decrease in the  
241 absolute FA level. The relative concentration of the sum of EPA+DHA, reflecting the  
242 endogenous n3-PUFA status, increased 2.5- and 3.7-fold in the kidney and whole blood,  
243 respectively (Fig. 2 B+D) indicating successful modulation of the n3-PUFA content by the  
244 supplementation.

245 Consistent to the changes in the PUFA pattern, levels of eicosanoids and other oxylipins derived  
246 from ARA, EPA and DHA were massively altered in response to the STD+n3 diet, while  
247 concentrations of oxylipins in the plasma and kidney were almost similar in the IRI treated group  
248 compared to the sham group both fed with the STD diet (Fig. 3). Comparing levels of individual  
249 oxylipins 24 h after IRI a similar trend was observed for free metabolites in the plasma as well as  
250 for free and esterified oxylipins in kidney tissue, though levels of esterified oxylipins were  
251 massively higher than the respective free mediators: Overall STD+n3 diet led to a significant  
252 decrease of ARA derived metabolites from all major formation pathways while EPA and DHA  
253 derived oxylipins were significantly elevated. Especially, regarding levels of free prostanoids in  
254 the kidney tissue, levels of ARA derived prostaglandins such as pro-inflammatory PGE<sub>2</sub> or the  
255 prostanoid TxB<sub>2</sub> were elevated following IRI compared to sham, while STD+n3 feeding caused  
256 their significant decrease and concomitant formation of the less potent EPA derived  
257 counterparts, i.e. PGE<sub>3</sub> and TxB<sub>3</sub> (Fig. 3 B). Amongst others in the circulation as well as in the

258 kidney also a significant increase of 17-HDHA and 18-HEPE, precursors for the formation of pro-  
259 resolving mediators (SPM), was observed in response to STD+n3. However, except for DHA  
260 derived protectin D1 (PD1) and its isomer PDX no signals above the lower limit of quantification  
261 (LLOQ), i.e. 0.25-2 nmol/kg in kidney tissue and 0.07-0.56 nM in plasma, were detected for all  
262 SPM. LC-MS/MS signals for PD1 indicated relevant apparent concentrations in plasma and  
263 kidney of STD+n3 fed mice (Fig, 3, SI Fig. S1). However, the ratios between monitored  
264 transitions differed significantly between the standard and the biological samples (Fig. S1).  
265 Similar to enzymatically formed oxylipins, levels of prostaglandin-like autoxidation products  
266 formed esterified in phospholipids were massively altered by n3-PUFA feeding. Total levels of  
267 ARA derived 5(*R,S*)-F<sub>2t</sub>-IsoP were decreased, while EPA and DHA derived 5(*R,S*)-5-F<sub>3t</sub>-IsoP  
268 and 4(*R,S*)-4-F<sub>4t</sub>-NeuroP were elevated in response to STD+n3. Interestingly, levels of these  
269 peroxidation products were lower following IRI compared to sham on the same STD diet (Fig.  
270 S2).

### 271 **Renal function, renal morphology and inflammation**

272 Despite successful n3-PUFA supplementation indicated by the changes of PUFA and oxylipin  
273 patterns renal function impairment was similar in both groups. IRI caused a 6-fold elevation of  
274 serum creatinine (Fig. 4 I). In line with renal function deterioration, renal damage measured by  
275 AKI score was similar in both groups (Fig. 4 A, D, G). Furthermore, Gr-1 positive neutrophil  
276 infiltration showed moderate to severe leukocyte infiltration in both groups. On mRNA-level the  
277 pro-inflammatory cytokines IL-6 and MCP-1 were elevated in both groups. The STD+n3 group  
278 even showed a trend towards higher values compared to the STD group (Fig. 4 C, F).

### 279 **Tubular function and heme oxygenase-1 expression**

280 Alpha-1 microglobulin (A1M) is synthesized in the liver, filtered by the glomeruli and reabsorbed  
281 by proximal tubuli. In healthy kidneys A1M is present in vesicles in the cytoplasm of 60-70% of

282 the tubuli. Upon AKI tubuli have a breakdown in energy metabolism and impaired transport  
283 function leading to lower cytoplasm concentration of A1M [48]. Due to IRI A1M expression  
284 decreased but was significantly higher in the STD+n3 group indicating better preservation of the  
285 tubular homeostasis and energy metabolism (Fig. 5 A, C, F). Heme oxygenase-1 (HO-1) is a  
286 renoprotective enzyme which is upregulated after AKI and mediates healing [49]. HO-1  
287 expression was significantly higher in STD+n3 fed mice (Fig. 5 B, D, G). Liver enzymes were  
288 elevated after IRI due to distant organ injury [50]. STD+n3 treatment caused less elevation of  
289 liver enzymes compared to STD (Fig. 5 E). Overall, we observed some protective effects by the  
290 STD+n3 diet in the context of renal IRI (Fig. 5).

291

292 **Discussion**

293 Renal IRI is a common cause of AKI which is associated with high morbidity and mortality rates  
294 [7]. Particularly in the context of solid organ transplantations IRI is inevitable contributing to  
295 impaired allograft function [51]. Cardiac surgery in elderly patients with slightly decreased renal  
296 function has incidence rates of AKI of ~30% [52]. In the context of scheduled surgeries a dietary  
297 intervention would be possible, while the patients are waiting for the surgery. Beneficial effects of  
298 n3-PUFA supplementation have been reported, however in the context of kidney transplantation  
299 the outcome is inconsistent [26].

300 In the present study we investigated if dietary n3-PUFA supplementation improves renal function  
301 impairment in an ischemia induced AKI model following renal IRI in mice [42]. The control group  
302 received a chow, reflecting the omega-6 rich western diet [43] containing 10% linoleic acid rich  
303 sunflower oil (STD). For the investigation of potential beneficial effects of n3-PUFA  
304 supplementation on the base of a western diet this chow was enriched with n3-PUFA (1% EPA  
305 and 1% DHA; STD+n3) as previously described [22]. In order to ensure a maximal modulation of  
306 the tissue fatty acid composition by the dietary PUFA supplementation a pre-feeding period of 14  
307 days was included prior induction of renal IRI. After 14 days of feeding the STD+n3 chow, the  
308 relative fatty acid pattern in the kidney, e.g. relative levels of the sum of saturated fatty acids,  
309 monounsaturated fatty acids, n6-PUFA and n3-PUFA (38.3%, 10.3%, 24.6% and 26.9%  
310 respectively), was comparable to the relative levels observed after 45 days of feeding the same  
311 diet to mice (38.1%, 13.0%, 25.2% and 23.9% respectively) [22] indicating that a steady-state in  
312 the modulation of the kidney fatty acid composition by the feeding was reached after 14 days.  
313 This is also consistent with results from feeding fish oil to mice where a steady state of the PUFA  
314 status in heart and brain tissue was reached between 1 and 2 weeks [53]. Overall, feeding of the  
315 STD+n3 diet led to a massive elevation of absolute and relative levels of n3-PUFA (18:4n3,  
316 20:4n3, 20:5n3, 22:5n3, 22:6n3) with simultaneous decrease in n6-PUFA (18:3n6, 20:2n6,  
317 20:3n6, 20:4n6, 22:4n6, 22:5n6) in the circulation and in the kidney. These changes are in line

318 with previous reports showing extensive modulation of PUFA composition in blood  
319 compartments and tissues by n3-PUFA feeding [22, 54-58] or by endogenous n3-PUFA  
320 accumulation in transgenic fat-1 mice [22, 54, 59]. Compared to the STD chow, in response to  
321 the STD+n3 feeding the relative content of the main long-chain PUFA ARA, EPA and DHA in the  
322 kidney changed from 16.4% to 8.6% (0.5 fold), 0.04% to 4.1% (99 fold) and 10.1% to 21.0% (2  
323 fold), respectively. Similar alterations in the renal fatty acid composition were also observed in  
324 mice and rats fed chow containing 1-3% each EPA and DHA (in each case in almost equal  
325 amount) [22, 55-57]. Relative levels in the kidney in response to STD+n3 are similar to earlier  
326 findings for n3-PUFA feeding in rodents ranging for EPA from 1-4.6% in mice and from 5.2-9.7%  
327 in rats and for DHA from 6.7-24.6% in mice and from 5.5-7.8% in rats depending primarily on the  
328 diet composition (e.g. ratio of n3/n6-PUFA). The differences in relative renal levels of EPA and  
329 DHA between mice and rats mainly originate from differing PUFA compositions under basal  
330 conditions (steady-state without n3-PUFA supplementation) [60]. The relative renal PUFA  
331 composition in response to STD+n3 was similar to the relative amount in murine renal  
332 phospholipids, especially phosphatidylcholines (9-12.4, 2.3-7.5 and 10-18.1% of ARA, EPA and  
333 DHA respectively) observed after feeding fish oil supplemented chow [54, 58], reflecting that  
334 phospholipids as main membrane constituents are dominant lipids in the kidney [61, 62].  
335 It should be noted, that the dietary supplementation by STD+n3 led to a more pronounced  
336 modulation of the n3-PUFA pattern compared to endogenous synthesis in transgenic fat-1 mice  
337 [22, 54]: The relative levels in transgenic fat-1 mice for ARA, EPA and DHA of 20.3%, 1% and  
338 12.1% in the kidney [22] and of 11.3%, 1%, 12.9% in kidney phospholipids, respectively [54]  
339 reveal that relative levels reached by STD+n3 feeding are around 4.1 fold and 1.7 fold higher for  
340 EPA and DHA respectively.  
341 The successful modulation of the endogenous n3-PUFA status by the feeding was also clearly  
342 indicated by the pronounced changes in %EPA+DHA levels in blood. In whole blood, which is  
343 dominated by the PUFA of erythrocytes, in response to the STD+n3 diet 3.7 fold higher levels of

344 %EPA+DHA were observed compared to the STD diet ( $4.5 \pm 0.1$  in STD vs.  $16.9 \pm 0.3\%$  in  
345 STD+n3). Extrapolating from mice to man, these concentrations reflect a very high endogenous  
346 n3-PUFA status, e.g. determined by the omega-3 index, i.e. the relative content of EPA+DHA in  
347 membranes of erythrocytes. With a value of 16.9% the endogenous n3-PUFA levels reached in  
348 response to the STD+n3 feeding are significantly higher than those which efficiently reduce  
349 overall mortality and the risk for cardiovascular diseases (i.e. an omega-3 index  $\geq 8$ ) in men [20,  
350 21]. In contrast, the STD diet reflects the “western-diet” causing a low omega-3 index, even in  
351 mice, which more efficiently convert short chain n3-PUFA to long chain n3-PUFA than men [63].  
352 Thus, it can be concluded that the n3-PUFA feeding strategy used in this study reflects the  
353 maximal shift in the PUFA pattern which can be reached. On a side note, a well described  
354 clinical effect of n3-PUFA is a reduced serum triglyceride concentration [64, 65]. Indeed, the total  
355 FA and thus TG concentration in whole blood was reduced in the animals receiving the STD+n3  
356 compared to the STD diet (Fig. 2).

357 It is believed that a major portion of beneficial effects of n3-PUFA are mediated by changes in  
358 the pattern of oxygenated PUFA, i.e. eicosanoids and other oxylipins [27, 29]. As eicosanoids  
359 exert important functions in renal (patho-)physiology including regulation of renal blood flow,  
360 glomerular filtration rate and tubular transport function [34-37] modulation of the oxylipin pattern  
361 by n3-PUFA may also impact kidney function in renal IRI.

362 Reflecting changes in the PUFA composition, the feeding strategy used in this study resulted in  
363 massive alterations of oxylipins in blood and renal tissue from all major formation pathways  
364 (COX, LOX, CYP as well as non-enzymatic): ARA derived eicosanoids were significantly  
365 decreased while EPA and DHA derived oxylipins were significantly elevated in response to  
366 STD+n3 feeding (Fig. 3). Reduction of ARA derived eicosanoids was similar in plasma and  
367 kidney (free and total) for all investigated pathways with 40-75% lower levels after STD+n3  
368 compared to STD feeding. Similarly, DHA derived oxylipins were 1.5-3 fold increased in the  
369 kidney, however a more pronounced elevation (7-12 fold) was observed for DHA derived

370 products from 12/15-LOX as well as CYP in plasma. In contrast, EPA derived metabolites were  
371 increased to a greater extent (12-53 fold) and in several cases were below the LLOQ in STD  
372 while clearly elevated in STD+n3 fed mice. These changes are overall consistent with earlier  
373 studies after n3-PUFA (EPA and DHA) supplementation in mice [13, 22, 54, 66-69], rats [55, 66,  
374 70-72] and humans [67, 73-75].

375 The strongest decrease was observed in kidney levels of ARA derived prostanoids, i.e. PGE<sub>2</sub>  
376 (250 vs. 62 nmol/kg) and TxB<sub>2</sub> (6.9 vs <1.2 nmol/kg) and similar to earlier studies with EPA and  
377 DHA supplemented chow in mice [54, 66, 69]. Though respective EPA derived counterparts  
378 were simultaneously increased resulting levels were considerably lower compared to their  
379 corresponding ARA derived prostanoids which is in line with their lower conversion rate by COX  
380 in addition to a simple PUFA competition [76]. Considering that EPA derived prostanoids are  
381 less bioactive, e.g. PGE<sub>3</sub> possesses less affinity towards EP receptors and exhibits lower  
382 potency regarding second messenger release [77] and also have been shown to cause less  
383 secretion of pro-inflammatory IL-6 [78] compared to PGE<sub>2</sub>, this clear shift in the renal prostanoid  
384 pattern suggests that STD+n3 can contribute to a lower inflammatory status.

385 Regarding CYP derived metabolites a massive increase in levels of free terminal epoxy-PUFA  
386 derived from EPA and DHA, i.e. 17(18)-EpETE and 19(20)-EpDPE, was observed especially in  
387 plasma, concomitant with a relatively marked decrease (16 vs 5 nmol/kg in kidney and 1.3 vs  
388 0.4 nM in plasma) in ARA derived 14(15)-EpETrE. Besides displacement of ARA by the n3-  
389 PUFA in response to the STD+n3 feeding this profound increase in n3-PUFA derived epoxy-  
390 PUFA can be explained by equal or higher conversion rates of DHA and EPA by several CYP  
391 isoforms in comparison to ARA [55, 79]. This marked elevation in n3-PUFA derived terminal  
392 epoxy-PUFA in plasma following STD+n3 is similar to their clear increase observed in human  
393 plasma after fish oil supplementation [73, 80] supporting their ability to function as indicator for  
394 n3-supplementation as has been suggested [39]. Studies regarding the physiological role of  
395 epoxy-PUFA in the kidney are mainly focused on ARA derived EpETrE and comprise, e.g.

396 regulation of renal blood flow, inhibition of tubular sodium transport thereby promotion of salt  
397 excretion as well as anti-inflammatory effects [81]. Treatment of renal IRI in mice with an  
398 inhibitor of the soluble epoxide hydrolase have been shown to ameliorate kidney injury  
399 suggesting that epoxy-PUFA have beneficial effects on kidney function in renal IRI [82].  
400 Similarly, n3-PUFA derived EpETE and EpDPE might impact renal physiology, e.g. owing to  
401 their higher potency regarding vasodilatory actions compared to EpETrE as well as similar anti-  
402 inflammatory effects [81]. Regarding epoxy-PUFA, 19(20)-EpDPE reduced kidney fibrosis in a  
403 murine unilateral ureteral obstruction (UUO) model [83]. In contrast, in renal IRI administration of  
404 19(20)-EpDPE led to aggravation of kidney damage in mice while 14(15)-EpETrE as well as  
405 DHA itself alleviated kidney injury in mice [84].

406 In the past two decades, a class of multiple hydroxylated PUFA were proposed which actively  
407 terminate inflammation, so-called specialized pro-resolving mediators (SPM). From DHA,  
408 D-series resolvins as well as protectins and maresins are formed, while EPA gives rise to E-  
409 series resolvins [38]. Because of their low biological levels, these mediators are difficult to  
410 analyze [40]. Consistent with earlier reports [22, 66, 70] feeding of the n3-PUFA EPA and DHA  
411 led to a massive increase (2-9 fold) in the precursors of SPM, i.e. 17-HDHA, 14-HDHA and 18-  
412 HEPE. For example, the 18-HEPE content in the kidney in the STD+n3 group was 9.7 nmol/kg  
413 while in the control group it was below the LLOQ (1 nmol/kg). However, except for PD1 all  
414 signals for SPM were below the LLOQ, i.e. in most cases below 2 nmol/kg kidney tissue. Thus, it  
415 has to be concluded that a 14-day feeding strategy with high n3-PUFA content (2%) does not  
416 increase the concentration of these mediators above 2 nmol/kg tissue or 0.56 nM blood. Either  
417 the SPM influence physiology in these picomolar, i.e. ppt (pg/g) concentrations or one has to  
418 conclude that they may not play a role in the physiological effects caused by n3-PUFA  
419 supplementation.

420 The signals for free PD1 in the kidney in response to STD+n3 are in a similar range to a  
421 previous report detecting PD1 after fish oil supplementation in mice undergoing renal IRI [13].

422 Analysis of total oxylipins in the kidney suggested even higher levels of PD1 (SI, Fig. S1). As  
423 internationally agreed for LC-MS compound identification (e. g. for pesticides [85]), we evaluated  
424 two mass transitions, i.e.  $m/z$  359  $\rightarrow$  153 [13, 86-88] and 359  $\rightarrow$  206 [86, 89, 90] which also  
425 have been previously used for PD1 detection. Particularly in the analysis of the total PD1  
426 concentration in the kidney massive divergence in the area ratio of the two transitions (compared  
427 to authentic PD1) results (SI Fig. S1). This clearly suggests that the signals result from isobaric  
428 interferences from the sample matrix and not from PD1 (SI Fig. S1). For free oxylipins in the  
429 kidney no signal was observed for  $m/z$  359  $\rightarrow$  206 and thus, it is even more clear that the signal  
430 of PD1 results from interfering matrix and not from the SPM. Isobaric interferences at the  
431 retention time of PD1 in reversed-phase HPLC at  $m/z$  359  $\rightarrow$  153 causing an apparent PD1  
432 concentration have been recently also reported for plasma samples after storage [91]. By  
433 contrast, the relative intensities of the signals of the PD1-isomer PDX showed identical ratios  
434 between standard and samples (SI Fig. S1). Overall our data do not support the earlier  
435 described renal formation of PD1 in a mouse model of AKI following feeding a slightly lower  
436 dose of an n3-PUFA (1.4% n3-PUFA in chow compared to 2% in this study) [13].  
437 N3-PUFA are prone to oxidation and may lead to increased oxidative stress. Indeed, in humans  
438 receiving a very high dose of DHA (1.6 g/d) urinary 15-F<sub>2t</sub>-IsoP, a marker of oxidative stress,  
439 was increased [92]. This could be even more important in ischemic injury were enhanced  
440 oxidative stress plays a key role in pathophysiology [6]. However, our data reveal, that the  
441 STD+n3 group did not show elevated IsoP levels in the kidney as well as in plasma following IRI  
442 compared to the STD diet (Fig. 3, SI Fig. S2). Similarly, lower levels of the autoxidation marker  
443 malondialdehyde were observed in the kidney tissue of rats treated with n3-PUFA after renal IRI  
444 [11, 12]  
445 Altogether feeding of STD+n3 led to a maximal modulation in n3-PUFA and its lipid mediators  
446 dominated by n3-PUFA derived oxylipins. Thus, the feeding strategy allows to evaluate the  
447 effects of maximal dietary n3-PUFA modulation based on a western diet on acute kidney injury.

448 Despite the clear effects on oxylipins the STD+n3 diet did not result in improvement of overall  
449 renal function or reduction of pro-inflammatory cytokine production or leukocyte infiltration 24 h  
450 after IRI. However, there were measurable effects on protective molecular mechanisms such as  
451 up-regulation of HO-1. It has been shown that HO-1 fosters renal regeneration and healing in  
452 several models of renal injury [45, 93, 94]. In addition, the tubular transport function which  
453 depends on intact energy metabolism of the tubuli was preserved as shown by A1M staining.  
454 There was a direct correlation in previous studies between higher A1M expression and less  
455 tissue edema measured by functional MRI [48]. In the current study there was also less ALT  
456 elevation which is a marker for impaired liver function. It is well known that AKI causes distant  
457 organ injury via enhanced leukocyte infiltration into other organs such as liver, lung, brain, gut  
458 [50, 95]. Mechanisms are multifactorial such as release of cytokines and inflammatory  
459 mediators, increase in oxidative stress, activation of various immune cells, neutrophil  
460 extravasation, generalized endothelial injury, increased vascular permeability and tissue edema  
461 formation [96]. In a mouse model of liver IRI also beneficial effects with less tissue damage has  
462 been attributed to n3-PUFA treatment [97]. It is conceivable that the moderate beneficial effects  
463 would improve patient outcome in longer follow-up by reducing the general inflammatory status.  
464 In a previous study on renal IRI beneficial effects of dietary n3-PUFA supplementation have  
465 been reported [13]. However, the severity of AKI might differ between the studies. In the study  
466 by Hassan et al. creatinine elevation was only 2-3-fold which is less than in our study (6-fold)  
467 and might reflect a milder AKI model. In the clinical context interesting and relevant benefits from  
468 n3-PUFA diets were observed, e.g. they seemed to reduce cardiovascular mortality in patients  
469 with chronic kidney disease [98]. In addition, another study showed that n3-PUFA reduced blood  
470 pressure and triglyceride levels in patients with CKD stage 3-4 in a placebo-controlled  
471 intervention study [99]. Since AKI has a high risk of progression to CKD there might be later  
472 beneficial effects on clinical outcome which are missed by the nature of the current study design

473 with a 24 hour follow-up. Further investigations with in depth analysis of oxylipin levels also at  
474 later time points of disease progression might be of interest and shall be addressed in the future.

475

## 476 **Conclusion**

477 In the present study supplementation of a western-style, i.e. linoleic rich diet, with the long chain  
478 n3-PUFA EPA and DHA led to a pronounced modulation of the PUFA and oxylipin pattern.

479 However, in a murine model of moderate AKI pre-feeding of n3-PUFA did not attenuate renal  
480 function impairment, morphological renal damage and inflammation characterized by pro-  
481 inflammatory cytokine (IL-6 and MCP-1) mRNA up-regulation and neutrophil infiltration.

482 However, also beneficial effects were seen such as preservation of tubular transport function  
483 indicated by enhanced alpha-1 microglobulin (A1M) expression in cortical proximal tubular  
484 epithelial cells and enhanced up-regulation of tubular heme oxygenase-1 (HO-1) expression  
485 which has been correlated to improved renal regeneration [45].

486 Overall it is concluded that - despite a maximal elevation of endogenous n3-PUFA and n3-PUFA  
487 derived oxylipin levels - n3-PUFA supplementation did not result in attenuation of renal function  
488 impairment or acute kidney injury within 24 hours in this model.

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803

804 **Figure Captions**

805 **Fig. 1:** Simplified overview of the biosynthesis of arachidonic acid (20:4n6, ARA) from linoleic  
806 acid (18:2n6, LA) and eicosapentaenoic acid (20:5n3, EPA) and docosahexaenoic acid (20:6n3,  
807 DHA) from  $\alpha$ -linolenic acid (18:3n3, ALA) and selected pathways for their oxygenation within the  
808 ARA cascade.

809 COX – cyclooxygenase, LOX – lipoxygenase, CYP – cytochrome P450 monooxygenase, Tx –  
810 thromboxane; HETE – hydroxyeicosatetraenoic acid; HEPE – hydroxyeicosapentaenoic acid;  
811 HDHA – hydroxydocosahexaenoic acid; EpETrE – epoxyeicosatrienoic acid; EpETE –  
812 epoxyeicosatetraenoic acid; EpDPE – epoxydocosapentaenoic acid; IsoP – isoprostanes;  
813 NeuroP – neuroprostanes; Rv – resolvin; Lx – lipoxin; PD – protectin; MaR - maresin

814  
815 **Fig. 2:** Fatty acid composition in **(A)** kidney tissue and **(C)** whole blood 24 h after renal IRI in  
816 mice was shifted towards higher levels of n3-PUFA, while n6-PUFA decreased after 14 days of  
817 feeding a sunflower oil based diet enriched with EPA and DHA (STD+n3) compared to the same  
818 diet without EPA and DHA (STD). Consistently relative levels of EPA and DHA of all detected FA  
819 in **(B)** kidney tissue and **(D)** whole blood were significantly higher in mice on the STD+n3 diet.  
820 Shown are mean  $\pm$  SEM, n=14, \*\*p<0.01, \*\*\*p<0.001.

821  
822 **Fig. 3:** Concentration of free oxylipins in **(A)** plasma and **(B)** kidney tissue as well as **(C)** total,  
823 i.e. free and esterified oxylipins in kidney tissue. Shown are concentrations of selected (iso-)  
824 prostanoids, 5-LOX, 12/15-LOX, CYP4 and CYP2 products of ARA, EPA and DHA as well as  
825 18-HEPE and PD1. Except for the sham group all patterns are determined in mice 24 h after  
826 renal IRI on a STD or STD+n3 diet. Feeding of the STD+n3 led to an increase of EPA and DHA  
827 derived lipid mediators while ARA derived oxylipins were decreased. Shown are mean  $\pm$  SEM  
828 (sham n=5, (A) STD: n=19, STD+n3: n=18; (B) STD: n=13, STD+n3: n=14; (C) STD: n=7,  
829 STD+n3: n=7). The lower limit of quantification (LLOQ) for those oxylipins is indicated by a  
830 dashed line in case it was not exceeded in >50% of the samples per group. Statistical  
831 differences were determined between STD and STD+n3.

832  
833 **Fig. 4:** Acute kidney injury and inflammation. Renal damage after IRI was comparable in STD  
834 and STD+n3 fed mice **(A, D, G;** STD: n=18, STD+n3: n=21). Neutrophil infiltration to the outer  
835 medulla was present in both groups without differences **(B, E, H;** STD: n=18, STD+n3: n=21).  
836 Renal function was significantly impaired after IRI in both groups **(I;** STD: n=19, STD+n3: n=21,  
837 sham n=5). Relative expression of **(C)** IL-6 and **(F)** MCP-1 mRNA in kidney tissue was  
838 significantly up-regulated at 24 h after IRI in both groups without differences (STD: n=7,  
839 STD+n3: n=7). Shown are mean  $\pm$  SEM.

840  
841 **Fig. 5:** Tubular transport and tubular regeneration. A1M expression was significantly higher after  
842 STD+n3 diet which indicates preserved tubular transport function **(A, C, F,** STD: n=18, STD+n3:

843 n=14). In addition, HO-1 expression was significantly upregulated in the STD+n3 fed group (**B**,  
844 **D**, **G**; STD: n=7, STD+n3 n=7) which correlates with tubular regeneration. Noteworthy, the  
845 elevation of the liver enzyme ALT was attenuated (**E**) by the STD+n3 diet and could point  
846 towards reduced distant organ injury (STD: n=19, STD+n3 n=21, sham n=5). Shown are mean  $\pm$   
847 SEM, \* $p < 0.05$ .

848