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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

Katharina Rund^{1#}, Shu Peng^{2,3#}, Robert Greite², Cornelius Claaßen¹, Fabian Nolte¹, Camille Oger⁴, Jean-Marie Galano⁴, Laurence Balas⁴, Thierry Durand⁴, Rongjun Chen², Faikah Gueler^{2**}, Nils Helge Schebb^{1**}

¹ Chair of Food Chemistry, Faculty of Mathematics and Natural Sciences, University of Wuppertal, Wuppertal, Germany

² Nephrology, Hannover Medical School, Hannover, Germany

³ Department of Thoracic surgery, Tongji hospital, Tongji medical college, Huazhong University of Science and Technology

⁴ Institut des Biomolécules Max Mousseron (IBMM), UMR 5247 CNRS, Université de Montpellier, ENSCM, France

Authors contributed equally

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

Highlights

- Maximal modulation of n3-PUFA and their oxylipins by long chain n3-PUFA feeding
- Apparent PD1 signals in LC-MS/MS analysis seem to result from isobaric interference
- Isoprostanes follow the modulation of parent PUFA in response to feeding: Increase of n3-isoprostanes / decrease of n6-isoprostanes
- Dietary n3-PUFA did not improve overall renal function following ischemic reperfusion injury

- Moderate protection of tubular function by dietary n3-PUFA and its oxylipins

Abstract

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

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

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

Discussion: We showed that n3-PUFA pre-treatment did not affect overall renal function or renal inflammation in a mouse model of moderate ischemia induced AKI, but tubular transport was improved. In conclusion, n3-PUFA food supplementation altered the oxylipin levels significantly but did not protect from renal function deterioration or attenuate ischemia induced renal inflammation.

Introduction

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

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

Established strategies to prevent or attenuate AKI even in scheduled surgeries such as cardiac valve replacement are lacking. Several studies discussed a beneficial role of omega-3 polyunsaturated fatty acids (n3-PUFA) in the context of kidney diseases [10-19]. For the prevention of cardiovascular diseases the nutritional-status of n3-PUFA is a well-established risk

factor [20, 21]. The higher the incorporation of n3-PUFA, determined by the relative content of EPA and DHA in the cells, typically red blood cells [20, 22], the lower the risk for cardiovascular diseases and mortality [23]. It has to be noted that n3-PUFA are essential constituents of the human diet and that the nutritional status and not the intake of n3-PUFA supplements is related to their physiological effects. Thus, if only the intake of n3-PUFA by supplementation is correlated to the risk for cardiovascular diseases the outcome is less clear [24, 25].

In the context of renal diseases effects of n3-PUFA supplementation on the outcome in kidney transplantation are inconsistent [26]. However, in some experimental AKI models the administration of n3-PUFA correlated with an improved kidney function [10-15].

The molecular mechanisms underlying beneficial effects of n3-PUFA include direct actions, e.g. binding to ion channels or transcription factors, e.g. NF κ B or PPAR γ , thereby reducing the expression of pro-inflammatory and activating the expression of anti-inflammatory genes, respectively. As constituents of membranes, e.g. predominantly in phospholipids, n3-PUFA also impact membrane structure and fluidity [27]. However, it is beyond doubt that a relevant part of the physiological effects of n3-PUFA are mediated by their oxygenated products, i.e. eicosanoids and other oxylipins [27-29]. In the so-called arachidonic acid (ARA) cascade PUFA serve as substrates for enzymatic and non-enzymatic conversion resulting in a multitude of lipid mediators from both n6- and n3-PUFA (Fig. 1) [27, 28, 30]. Enzymatic conversion of PUFA in the ARA cascade comprises three major pathways: (I) cyclooxygenases (COX) lead to the formation of prostanoids and thromboxanes, (II) cytochrome P450 monooxygenases (CYP) give rise to epoxy-PUFA and terminal hydroxy-PUFA and (III) lipoxygenases lead via hydroperoxy-PUFA to hydroxy-PUFA and leukotrienes [31]. Structurally similar to these enzymatically formed products oxidized PUFA also arise during non-enzymatic autoxidation, e.g. isoprostanes, which also have been shown to possess biological activity [32, 33]. In the kidney, oxylipins have important functions in (patho-)physiology, e.g. regulation of renal blood flow, glomerular filtration rate and tubular transport function [34-37]. As n3-PUFA compete with ARA for conversion dietary n3-PUFA

supplementation impacts the overall oxylipin profile resulting in a shift from predominantly pro-inflammatory, e.g. 2-series prostaglandins, towards n3-PUFA derived oxylipins with less potency or even anti-inflammatory properties [27]. Furthermore, in recent years activity of LOX with n3-PUFA have been related to the formation of multiple hydroxylated PUFA, e.g. resolvins, maresins and protectins which have been attributed to be actively involved in the resolution of inflammation [38], though several studies fail to detect them in biological samples [39, 40].

Changes in the oxylipin pattern in the context of kidney function in AKI have been sparsely investigated. Therefore, we used a well characterized model of ischemia induced AKI in mice [41, 42] to study the effect of dietary n3-PUFA (EPA and DHA) supplementation - based on a linoleic acid rich western-like diet [43] for 14 days prior to surgery - on the fatty acid composition and oxylipin pattern in blood and tissue in comparison to renal damage and inflammation at 24 h after injury.

Materials and methods

Chemicals

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

Feeding experiment and renal ischemia reperfusion injury in mice

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

in the supplementary material (SI, Tab. S1). During the whole feeding period every 2-3 days fresh chow was provided and animals had free access to the food and domestic quality drinking water. After 14 days of feeding, renal IRI was initiated in general isoflurane anesthesia (5% induction, 2% maintenance) combined with iv butorphanol as analgetic treatment. IRI was induced by transient bilateral renal pedicle clamping for 30 min using a non-traumatic vascular clamp [45]. Mice were sacrificed 24 h after reperfusion by deep general anesthesia and total body perfusion with ice cold PBS [45].

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

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

Histology and immunohistochemistry

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

5-25% of tubuli affected, 2 = moderate AKI with 26-50% of tubuli affected, 3 = severe AKI with 51-75% of the tubuli affected, 4 = very severe AKI with > 75% of tubuli affected.

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

Detection of pro-inflammatory cytokines by qPCR

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

Extraction and quantification of fatty acids

The fatty acid composition was analyzed in diluted whole blood (15 μ L blood + 75 μ L water) and kidney tissue (15 \pm 2 mg) as fatty acid methyl esters using gas chromatography with flame ionization detection (GC-FID) as described with slight modifications [46]. Briefly, 10 μ L internal standard (FAME C25:0, 750 μ M) was added to diluted whole blood and kidney tissue samples. Kidney tissue was homogenized in 300 μ L MeOH and 50 μ L water using two stainless steel beads (3 mm, 5 min, 25 Hz) with a vibration ball mill (MM 400, Retsch, Haan, Germany). Lipids were extracted with MeOH/methyl *tert*-butyl ether and the lipid extract was trans-esterified to fatty acid methyl esters using methanolic hydrogen chloride. The calculation of the absolute fatty acid concentrations and relative pattern was based on response factors as described [46]. The LLOQ was 0.5 μ g/ml for all FAME in the injected solution and the variability in quality control plasma was < 5% within batch.

Extraction and quantification of oxylipins

Oxylipins in plasma and kidney tissue were extracted using anion exchange Bond Elut Certify II SPE cartridges (Agilent, Waldbronn, Germany) as described with modifications [40, 44]. In the first step of oxylipin extraction 10 μ L antioxidant solution and internal standards were added to each sample.

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

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

For quantification of total, i.e. free and esterified oxylipins in kidney tissue (20 ± 2 mg) samples were homogenized in 400 μ L isopropanol and stored for 30 min at -80°C . Samples were centrifuged and the supernatant was hydrolyzed (300 μ L 1.5M KOH (75/25, MeOH/water, v/v), immediately neutralized with acetic acid, diluted with 2 mL sodium phosphate buffer (pH 5.5) and loaded on the preconditioned SPE cartridge.

The SPE procedure was carried out as described and samples were analyzed by LC-MS/MS (QTRAP, Sciex, Darmstadt, Germany) in scheduled selected reaction monitoring mode following negative electrospray ionization as described [40, 44]. The lower limit of quantification [40, 44] and the analytical variability [48] of the quantified oxylipins are summarized in the SI (Tab. S2).

Data analysis

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

Results

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

Blood and tissue pattern of fatty acids and their oxidative metabolites

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

Consistent to the changes in the PUFA pattern, levels of eicosanoids and other oxylipins derived from ARA, EPA and DHA were massively altered in response to the STD+n3 diet, while concentrations of oxylipins in the plasma and kidney were almost similar in the IRI treated group compared to the sham group both fed with the STD diet (Fig. 3). Comparing levels of individual oxylipins 24 h after IRI a similar trend was observed for free metabolites in the plasma as well as for free and esterified oxylipins in kidney tissue, though levels of esterified oxylipins were massively higher than the respective free mediators: Overall STD+n3 diet led to a significant decrease of ARA derived metabolites from all major formation pathways while EPA and DHA derived oxylipins were significantly elevated. Especially, regarding levels of free prostanoids in the kidney tissue, levels of ARA derived prostaglandins such as pro-inflammatory PGE₂ or the prostanoid TxB₂ were elevated following IRI compared to sham, while STD+n3 feeding caused their significant decrease and concomitant formation of the less potent EPA derived counterparts, i.e. PGE₃ and TxB₃ (Fig. 3 B). Amongst others in the circulation as well as in the kidney also a

significant increase of 17-HDHA and 18-HEPE, precursors for the formation of pro-resolving mediators (SPM), was observed in response to STD+n3. However, except for DHA derived protectin D1 (PD1) and its isomer PDX no signals above the lower limit of quantification (LLOQ), i.e. 0.25-2 nmol/kg in kidney tissue and 0.07-0.56 nM in plasma, were detected for all SPM. LC-MS/MS signals for PD1 indicated relevant apparent concentrations in plasma and kidney of STD+n3 fed mice (Fig. 3, SI Fig. S1). However, the ratios between monitored transitions differed significantly between the authentic standard and the biological samples (SI Fig. S1).

Similar to enzymatically formed oxylipins, levels of prostaglandin-like autoxidation products formed esterified in phospholipids were massively altered by n3-PUFA feeding. Total levels of ARA derived 5(*R,S*)-F_{2t}-IsoP were decreased, while EPA and DHA derived 5(*R,S*)-5-F_{3t}-IsoP and 4(*R,S*)-4-F_{4t}-NeuroP were elevated in response to STD+n3. Interestingly, levels of these peroxidation products were lower following IRI compared to sham on the same STD diet (SI Fig. S2).

Renal function, renal morphology and inflammation

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

Tubular function and heme oxygenase-1 expression

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

tubuli. Upon AKI tubuli have a breakdown in energy metabolism and impaired transport function leading to lower cytoplasm concentration of A1M [49]. Due to IRI A1M expression decreased but was significantly higher in the STD+n3 group indicating better preservation of the tubular homeostasis and energy metabolism (Fig. 5 A, C, F). Heme oxygenase-1 (HO-1) is a renoprotective enzyme which is upregulated after AKI and mediates healing [50]. HO-1 expression was significantly higher in STD+n3 fed mice (Fig. 5 B, D, G). Liver enzymes were elevated after IRI due to distant organ injury [51]. STD+n3 treatment caused less elevation of liver enzymes compared to STD (Fig. 5 E). Overall, we observed some protective effects by the STD+n3 diet in the context of renal IRI (Fig. 5).

Discussion

Renal IRI is a common cause of AKI which is associated with high morbidity and mortality rates [7]. Particularly in the context of solid organ transplantations IRI is inevitable contributing to impaired allograft function [52]. Cardiac surgery in elderly patients with slightly decreased renal function has incidence rates of AKI of ~30% [53]. In the context of scheduled surgeries a dietary intervention would be possible, while the patients are waiting for the surgery. Beneficial effects of n3-PUFA supplementation have been reported, however in the context of kidney transplantation the outcome is inconsistent [26].

In the present study we investigated if dietary n3-PUFA supplementation improves renal function impairment in an ischemia induced AKI model following renal IRI in mice [42]. The control group received a chow, reflecting the omega-6 rich western diet [43] containing 10% linoleic acid rich sunflower oil (STD). For the investigation of potential beneficial effects of n3-PUFA supplementation on the base of a western diet, this chow was enriched with n3-PUFA (1% EPA and 1% DHA; STD+n3) as previously described [22]. In order to ensure a maximal modulation of the tissue fatty acid composition by the dietary PUFA supplementation a pre-feeding period of 14 days was included prior induction of renal IRI. After 14 days of feeding the STD+n3 chow, the relative fatty acid pattern in the kidney, e.g. relative levels of the sum of saturated fatty acids,

monounsaturated fatty acids, n6-PUFA and n3-PUFA (38.3%, 10.3%, 24.6% and 26.9% respectively), was comparable to the relative levels observed after 45 days of feeding the same diet to mice (38.1%, 13.0%, 25.2% and 23.9% respectively) [22] indicating that a steady-state in the modulation of the kidney fatty acid composition by the feeding was reached after 14 days. This is also consistent with results from feeding fish oil to mice where a steady state of the PUFA status in heart and brain tissue was reached between 1 and 2 weeks [54]. Overall, feeding of the STD+n3 diet led to a massive elevation of absolute and relative levels of n3-PUFA (18:4n3, 20:4n3, 20:5n3, 22:5n3, 22:6n3) with simultaneous decrease in n6-PUFA (18:3n6, 20:2n6, 20:3n6, 20:4n6, 22:4n6, 22:5n6) in the circulation and in the kidney. These changes are in line with previous reports showing extensive modulation of PUFA composition in blood compartments and tissues by n3-PUFA feeding [22, 55-59] or by endogenous n3-PUFA accumulation in transgenic fat-1 mice [22, 55, 60]. Compared to the STD chow, in response to the STD+n3 feeding the relative content of the main long-chain PUFA ARA, EPA and DHA in the 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 fold), respectively. Similar alterations in the renal fatty acid composition were also observed in mice and rats fed chow containing 1-3% each EPA and DHA (in each case in almost equal amount) [22, 56-58]. Relative levels in the kidney in response to STD+n3 are similar to earlier findings for n3-PUFA feeding in rodents ranging for EPA from 1-4.6% in mice and from 5.2-9.7% in rats and for DHA from 6.7-24.6% in mice and from 5.5-7.8% in rats depending primarily on the diet composition (e.g. ratio of n3/n6-PUFA). The differences in relative renal levels of EPA and DHA between mice and rats mainly originate from differing PUFA compositions under basal conditions (steady-state without n3-PUFA supplementation) [61]. The relative renal PUFA composition in response to STD+n3 was similar to the relative amount in murine renal phospholipids, especially phosphatidylcholines (9-12.4, 2.3-7.5 and 10-18.1% of ARA, EPA and DHA respectively) observed after feeding fish oil supplemented chow [55, 59], reflecting that phospholipids as main membrane constituents are dominant lipids in the kidney [62, 63].

It should be noted, that the dietary supplementation by STD+n3 led to a more pronounced modulation of the n3-PUFA pattern compared to endogenous synthesis in transgenic fat-1 mice [22, 55]: The relative levels in transgenic fat-1 mice for ARA, EPA and DHA of 20.3%, 1% and 12.1% in the kidney [22] and of 11.3%, 1%, 12.9% in kidney phospholipids, respectively [55] reveal that relative levels reached by STD+n3 feeding are around 4.1 fold and 1.7 fold higher for EPA and DHA respectively.

The successful modulation of the endogenous n3-PUFA status by the feeding was also clearly indicated by the pronounced changes in %EPA+DHA levels in blood. In whole blood, which is dominated by the PUFA of erythrocytes, in response to the STD+n3 diet 3.7 fold higher levels of %EPA+DHA were observed compared to the STD diet (4.5 ± 0.1 in STD vs. $16.9 \pm 0.3\%$ in STD+n3). Extrapolating from mice to man, these concentrations reflect a very high endogenous n3-PUFA status, e.g. determined by the omega-3 index, i.e. the relative content of EPA+DHA in membranes of erythrocytes. With a value of 16.9% the endogenous n3-PUFA levels reached in response to the STD+n3 feeding are significantly higher than those which efficiently reduce overall mortality and the risk for cardiovascular diseases (i.e. an omega-3 index ≥ 8) in men [20, 21]. In contrast, the STD diet reflects the “western-diet” causing a low omega-3 index, even in mice, which more efficiently convert short chain n3-PUFA to long chain n3-PUFA than men [64]. Thus, it can be concluded that the n3-PUFA feeding strategy used in this study reflects the maximal shift in the PUFA pattern which can be reached. On a side note, a well described clinical effect of n3-PUFA is a reduced serum triglyceride concentration [65, 66]. Indeed, the total FA and thus TG concentration in whole blood was reduced in the animals receiving the STD+n3 compared to the STD diet (Fig. 2).

It is believed that a major portion of beneficial effects of n3-PUFA are mediated by changes in the pattern of oxygenated PUFA, i.e. eicosanoids and other oxylipins [27, 29]. As eicosanoids exert important functions in renal (patho-)physiology including regulation of renal blood flow, glomerular

filtration rate and tubular transport function [34-37] modulation of the oxylipin pattern by n3-PUFA may also impact kidney function in renal IRI.

Reflecting changes in the PUFA composition, the feeding strategy used in this study resulted in massive alterations of oxylipins in blood and renal tissue from all major formation pathways (COX, LOX, CYP as well as non-enzymatic): ARA derived eicosanoids were significantly decreased while EPA and DHA derived oxylipins were significantly elevated in response to STD+n3 feeding (Fig. 3). Reduction of ARA derived eicosanoids was similar in plasma and kidney (free and total) for all investigated pathways with 40-75% lower levels after STD+n3 compared to STD feeding. Similarly, DHA derived oxylipins were 1.5-3 fold increased in the kidney, however a more pronounced elevation (7-12 fold) was observed for DHA derived products from 12/15-LOX as well as CYP in plasma. In contrast, EPA derived metabolites were increased to a greater extent (12-53 fold) and in several cases were below the LLOQ in STD while clearly elevated in STD+n3 fed mice. These changes are overall consistent with earlier studies after n3-PUFA (EPA and DHA) supplementation in mice [13, 22, 55, 67-70], rats [56, 67, 71-73] and humans [68, 74-76].

The strongest decrease was observed in kidney levels of ARA derived prostanoids, i.e. PGE₂ (250 vs. 62 nmol/kg) and TxB₂ (6.9 vs <1.2 nmol/kg) and similar to earlier studies with EPA and DHA supplemented chow in mice [55, 67, 70]. Though respective EPA derived counterparts were simultaneously increased resulting levels were considerably lower compared to their corresponding ARA derived prostanoids which is in line with their lower conversion rate by COX in addition to a simple PUFA competition [77]. Considering that EPA derived prostanoids are less bioactive, e.g. PGE₃ possesses less affinity towards EP receptors and exhibits lower potency regarding second messenger release [78] and also have been shown to cause less secretion of pro-inflammatory IL-6 [79] compared to PGE₂, this clear shift in the renal prostanoid pattern suggests that STD+n3 can contribute to a lower inflammatory status.

Regarding CYP derived metabolites a massive increase in levels of free terminal epoxy-PUFA derived from EPA and DHA, i.e. 17(18)-EpETE and 19(20)-EpDPE, was observed especially in

plasma, concomitant with a relatively marked decrease (16 vs 5 nmol/kg in kidney and 1.3 vs 0.4 nM in plasma) in ARA derived 14(15)-EpETrE. Besides displacement of ARA by the n3-PUFA in response to the STD+n3 feeding this profound increase in n3-PUFA derived epoxy-PUFA can be explained by equal or higher conversion rates of DHA and EPA by several CYP isoforms in comparison to ARA [56, 80]. This marked elevation in n3-PUFA derived terminal epoxy-PUFA in plasma following STD+n3 is similar to their clear increase observed in human plasma after fish oil supplementation [74, 81] supporting their ability to function as indicator for n3-PUFA supplementation as has been suggested [39]. Studies regarding the physiological role of epoxy-PUFA in the kidney are mainly focused on ARA derived EpETrE and comprise, e.g. regulation of renal blood flow, inhibition of tubular sodium transport thereby promotion of salt excretion as well as anti-inflammatory effects [82]. Treatment of renal IRI in mice with an inhibitor of the soluble epoxide hydrolase have been shown to ameliorate kidney injury suggesting that epoxy-PUFA have beneficial effects on kidney function in renal IRI [83]. Similarly, n3-PUFA derived EpETE and EpDPE might impact renal physiology, e.g. owing to their higher potency regarding vasodilatory actions compared to EpETrE as well as similar anti-inflammatory effects [82]. Regarding epoxy-PUFA, 19(20)-EpDPE reduced kidney fibrosis in a murine unilateral ureteral obstruction (UUO) model [84]. In contrast, in renal IRI administration of 19(20)-EpDPE led to aggravation of kidney damage in mice while 14(15)-EpETrE as well as DHA itself alleviated kidney injury in mice [85].

In the past two decades, a class of multiple hydroxylated PUFA were proposed which actively terminate inflammation, so-called specialized pro-resolving mediators (SPM). From DHA, D-series resolvins as well as protectins and maresins are formed, while EPA gives rise to E-series resolvins [38]. Because of their low biological levels, these mediators are difficult to analyze [40]. Consistent with earlier reports [22, 67, 71] feeding of the n3-PUFA EPA and DHA led to a massive increase (2-9 fold) in the precursors of SPM, i.e. 17-HDHA, 14-HDHA and 18-HEPE. For example, the 18-HEPE content in the kidney in the STD+n3 group was 9.7 nmol/kg while in the control group it was below the LLOQ (1 nmol/kg). However, except for PD1 all signals for SPM were below the LLOQ,

i.e. in most cases below 2 nmol/kg kidney tissue. Thus, it has to be concluded that a 14-day feeding strategy with high n3-PUFA content (2%) does not increase the concentration of these mediators above 2 nmol/kg tissue or 0.56 nM blood. Either the SPM influence physiology in these picomolar, i.e. ppt (pg/g) concentrations or one has to conclude that they may not play a role in the physiological effects caused by n3-PUFA supplementation.

The signals for free PD1 in the kidney in response to STD+n3 are in a similar range to a previous report detecting PD1 after fish oil supplementation in mice undergoing renal IRI [13]. Analysis of total oxylipins in the kidney suggested even higher levels of PD1 (SI, Fig. S1). As internationally agreed for LC-MS compound identification (e. g. for pesticides [86]), we evaluated two mass transitions, i.e. m/z 359 \rightarrow 153 [13, 87-89] and 359 \rightarrow 206 [87, 90, 91] which also have been previously used for PD1 detection. Particularly in the analysis of the total PD1 concentration in the kidney massive divergence in the area ratio of the two transitions (compared to authentic PD1) results (SI Fig. S1). This clearly suggests that the signals result from isobaric interferences from the sample matrix and not from PD1 (SI Fig. S1). For free oxylipins in the kidney no signal was observed for m/z 359 \rightarrow 206 and thus, it is even more clear that the signal of PD1 results from interfering matrix and not from the SPM. Isobaric interferences at the retention time of PD1 in reversed-phase HPLC at m/z 359 \rightarrow 153 causing an apparent PD1 concentration have been recently also reported for plasma samples after storage [92]. By contrast, the relative intensities of the signals of the PD1-isomer PDX showed identical ratios between authentic standard and samples (SI Fig. S1). Overall our data do not support the earlier described renal formation of PD1 in a mouse model of AKI following feeding a slightly lower dose of n3-PUFA (1.4% n3-PUFA in chow compared to 2% in this study) [13].

N3-PUFA are prone to oxidation and may lead to increased oxidative stress. Indeed, in humans receiving a very high dose of DHA (1.6 g/d) urinary 15-F_{2t}-IsoP, a marker of oxidative stress, was increased [93]. This could be even more important in ischemic injury where enhanced oxidative stress plays a key role in pathophysiology [6]. However, our data reveal, that the STD+n3 group

did not show elevated IsoP levels in the kidney as well as in plasma following IRI compared to the STD diet (Fig. 3, SI Fig. S2). Similarly, lower levels of the autoxidation marker malondialdehyde were observed after renal IRI in the kidney tissue of rats treated with n3-PUFA [11, 12]. However, to comprehensively investigate oxidative stress caused by IRI and its modulation by n3-PUFA supplementation further autoxidation parameters, such as 8-hydroxyguanosine, glutathione, 4 hydroxy-nonenal and expression of (anti)oxidative stress response genes should be monitored in the future.

Altogether feeding of STD+n3 led to a maximal modulation in n3-PUFA and its lipid mediators dominated by n3-PUFA derived oxylipins. Thus, the feeding strategy allows to evaluate the effects of maximal dietary n3-PUFA modulation based on a western diet on acute kidney injury.

Despite the clear effects on oxylipins the STD+n3 diet did not result in improvement of overall renal function or reduction of pro-inflammatory cytokine production or leukocyte infiltration 24 h after IRI. However, there were measurable effects on protective molecular mechanisms such as up-regulation of HO-1. It has been shown that HO-1 fosters renal regeneration and healing in several models of renal injury [45, 94, 95]. In addition, the tubular transport function which depends on intact energy metabolism of the tubuli was preserved as shown by A1M staining. There was a direct correlation in previous studies between higher A1M expression and less tissue edema measured by functional MRI [49]. In the current study there was also less ALT elevation which is a marker for impaired liver function. It is well known that AKI causes distant organ injury via enhanced leukocyte infiltration into other organs such as liver, lung, brain, gut [51, 96]. Mechanisms are multifactorial such as release of cytokines and inflammatory mediators, increase in oxidative stress, activation of various immune cells, neutrophil extravasation, generalized endothelial injury, increased vascular permeability and tissue edema formation [97]. In a mouse model of liver IRI also beneficial effects with less tissue damage has been attributed to n3-PUFA treatment [98]. It is conceivable that the moderate beneficial effects would improve patient outcome in longer follow-up by reducing the general inflammatory status.

In a previous study on renal IRI beneficial effects of dietary n3-PUFA supplementation have been reported [13]. However, the severity of AKI might differ between the studies. In the study by Hassan et al. creatinine elevation was only 2-3-fold which is less than in our study (6-fold) and might reflect a milder AKI model. In the clinical context interesting and relevant benefits from n3-PUFA diets were observed, e.g. they seemed to reduce cardiovascular mortality in patients with chronic kidney disease [99]. In addition, another study showed that n3-PUFA reduced blood pressure and triglyceride levels in patients with CKD stage 3-4 in a placebo-controlled intervention study [100]. Since AKI has a high risk of progression to CKD there might be later beneficial effects on clinical outcome which are missed by the nature of the current study design with a 24 hour follow-up. Further investigations with in-depth analysis of oxylipin levels also at later time points of disease progression might be of interest and shall be addressed in the future.

Conclusion

In the present study supplementation of a western-style, i.e. linoleic rich diet, with the long chain n3-PUFA EPA and DHA led to a pronounced modulation of the PUFA and oxylipin pattern. However, in a murine model of moderate AKI pre-feeding of n3-PUFA did not attenuate renal function impairment, morphological renal damage and inflammation characterized by pro-inflammatory cytokine (IL-6 and MCP-1) mRNA up-regulation and neutrophil infiltration. However, also beneficial effects were seen such as preservation of tubular transport function indicated by enhanced alpha-1 microglobulin (A1M) expression in cortical proximal tubular epithelial cells and enhanced up-regulation of tubular heme oxygenase-1 (HO-1) expression which has been correlated to improved renal regeneration [45]. Thus n3-PUFA supplementation might lead to renal protection with respect to long-term damage.

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

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Figure Captions

Fig. 1: Simplified overview of the biosynthesis of arachidonic acid (20:4n6, ARA) from linoleic acid (18:2n6, LA) and eicosapentaenoic acid (20:5n3, EPA) and docosahexaenoic acid (22:6n3, DHA) from α -linolenic acid (18:3n3, ALA) and selected pathways for their oxygenation within the ARA cascade.

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

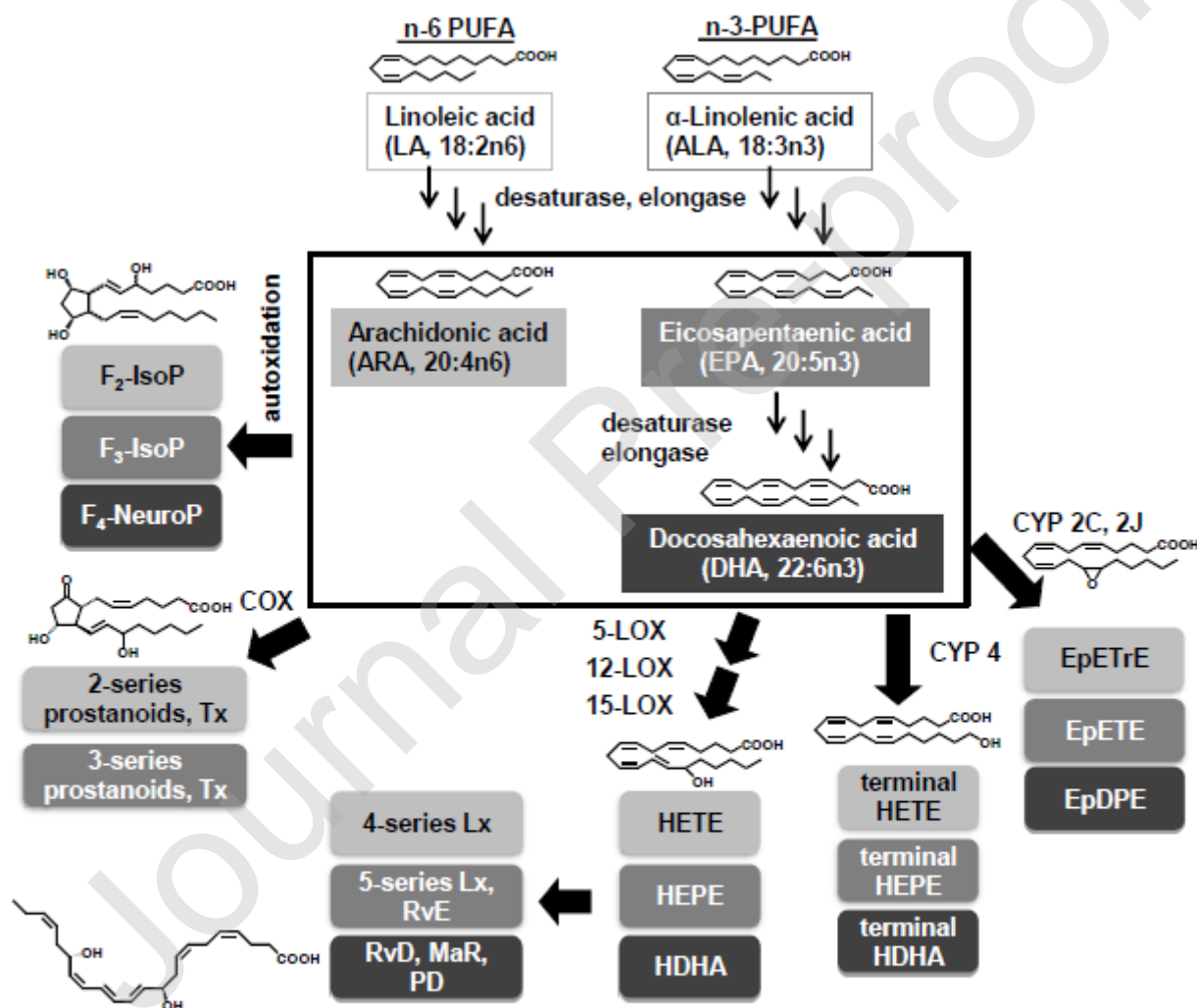


Fig. 2: Fatty acid composition in **(A)** kidney tissue and **(C)** whole blood 24 h after renal IRI in mice was shifted towards higher levels of n3-PUFA, while n6-PUFA decreased after 14 days of feeding a sunflower oil based diet enriched with EPA and DHA (STD+n3) compared to the same diet without EPA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

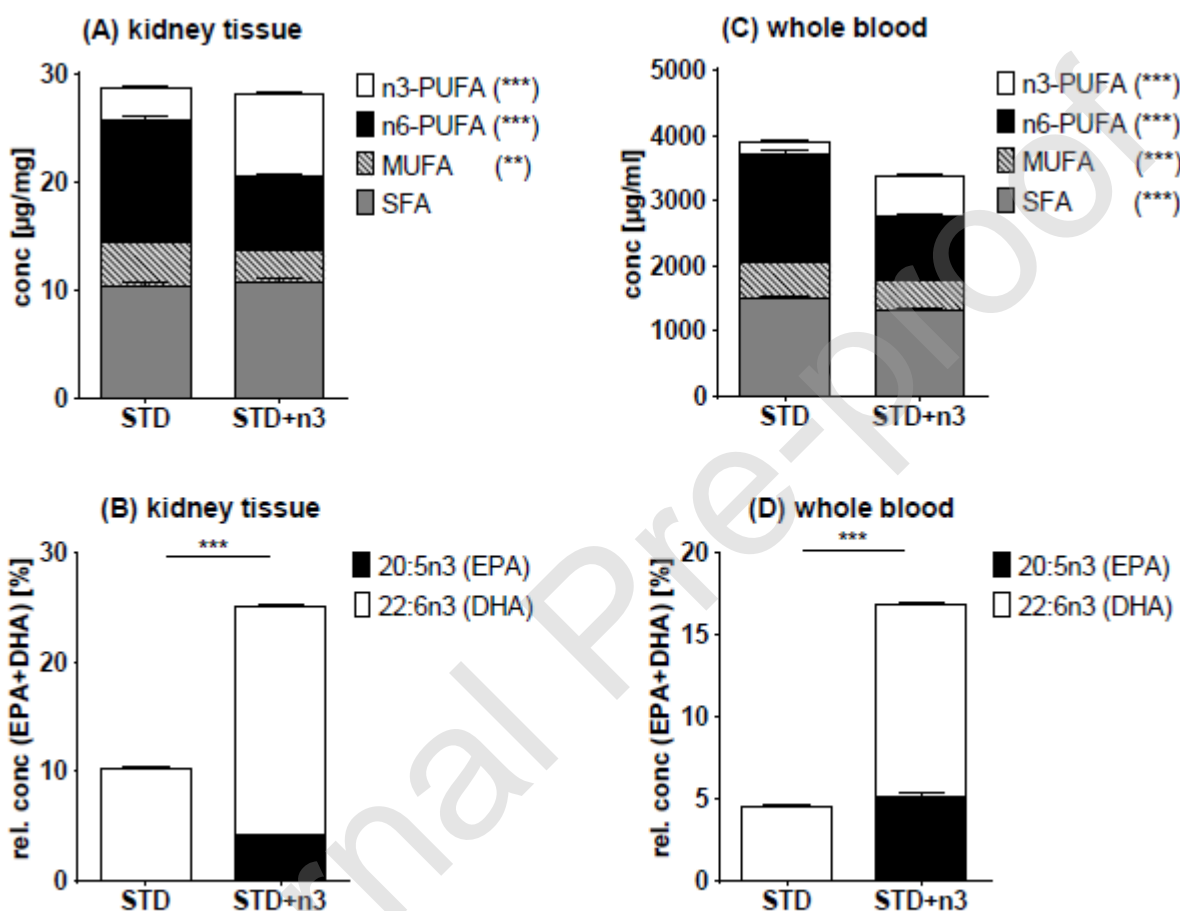


Fig. 3: Concentration of free oxylipins in **(A)** plasma and **(B)** kidney tissue as well as **(C)** total, i.e. free and esterified oxylipins in kidney tissue. Shown are concentrations of selected (iso-) prostanoids, 5-LOX, 12/15-LOX, CYP4 and CYP2 products of ARA, EPA and DHA as well as 18-HEPE and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

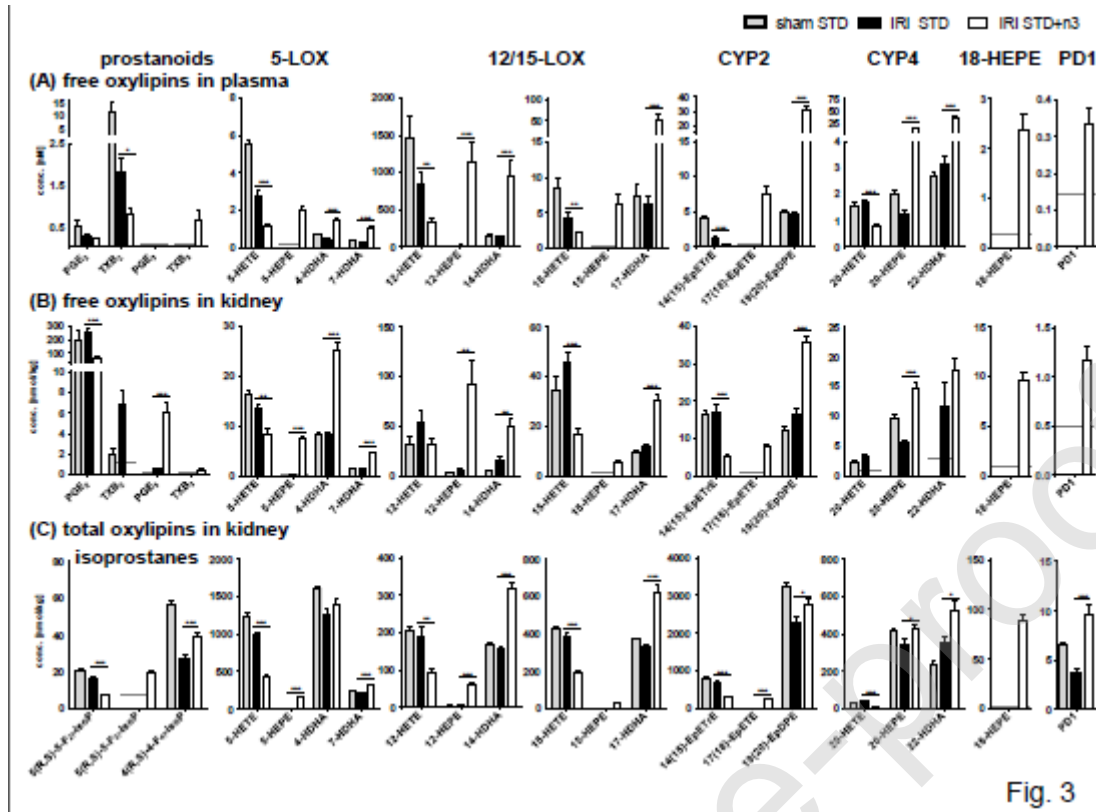


Fig. 3

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

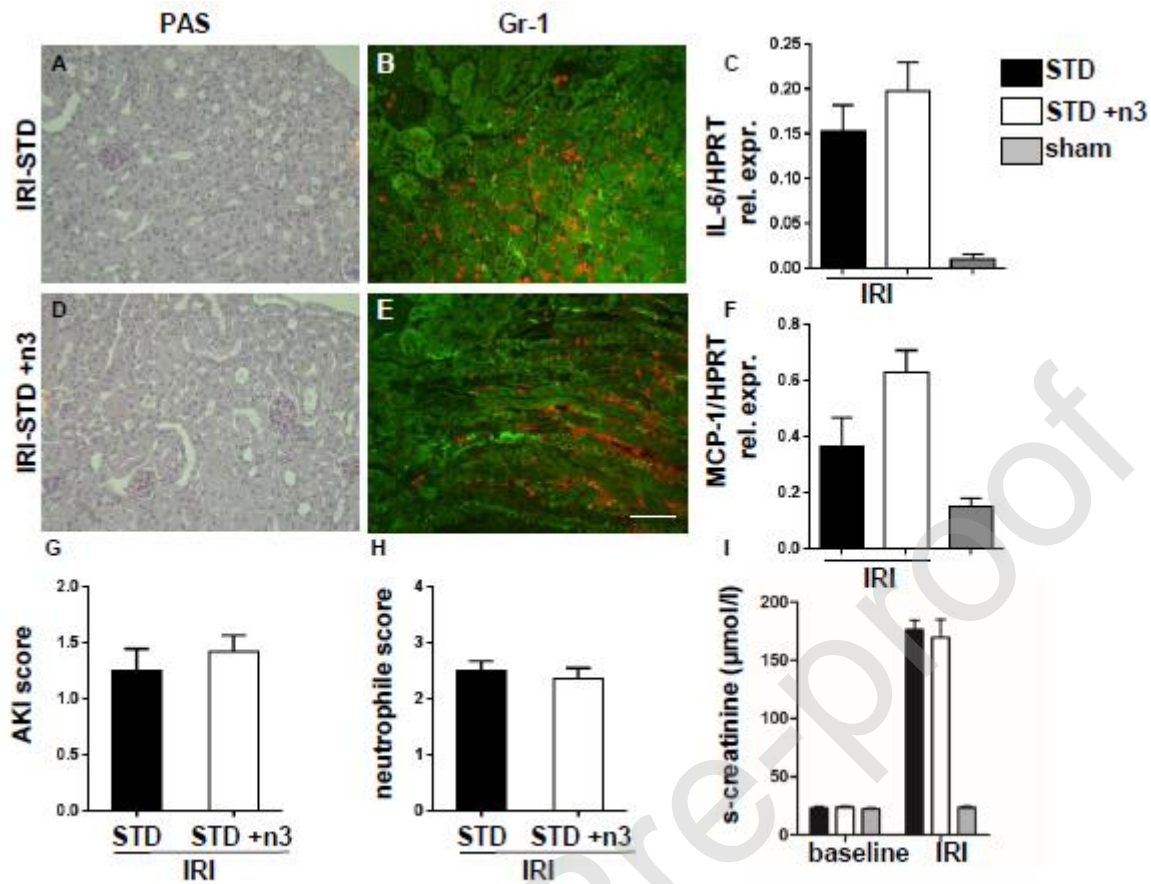


Fig. 5: Tubular transport and tubular regeneration. A1M expression was significantly higher after STD+n3 diet which indicates preserved tubular transport function (A, C, F, STD: n=18, STD+n3: n=14). In addition, HO-1 expression was significantly upregulated in the STD+n3 fed group (B, D, G; STD: n=7, STD+n3 n=7) which correlates with tubular regeneration. Noteworthy, the elevation of the liver enzyme ALT was attenuated (E) by the STD+n3 diet and could point towards reduced distant organ injury (STD: n=19, STD+n3 n=21, sham n=5). Shown are mean \pm SEM, * p <0.05.

