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Fish oil replacement prevents, while docosahexaenoic acid-derived protectin DX mitigates end-stage-renal-disease in atherosclerotic diabetic mice

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Abstract

Diabetic nephropathy (DN) remains the major cause of end-stage renal disease (ESRD). We used high-fat/high-sucrose (HFHS)-fed LDLr$^{-/-}$/ApoB$^{100/100}$ mice with transgenic overexpression of IGFII in pancreatic β-cells (LRKOB100/IGFII) as a model of ESRD to test whether dietary long chain omega-3 polyunsaturated fatty acids LCω3FA-rich fish oil (FO) could prevent ESRD development. We further evaluated the potential of docosahexaenoic acid (DHA)-derived pro-resolving lipid mediators, 17-hydroxy-DHA (17-HDHA) and Protectin DX (PDX), to reverse established ESRD damage. HFHS-fed vehicle-treated LRKOB100/IGFII mice developed severe kidney dysfunction leading to ESRD, as revealed by advanced glomerular fibrosis and mesangial expansion along with reduced percent survival. The kidney failure outcome was associated with cardiac dysfunction, revealed by reduced heart rate and prolonged diastolic and systolic time. Dietary FO prevented kidney damage, lean mass loss, cardiac dysfunction, and death. 17-HDHA reduced podocyte foot process effacement while PDX treatment alleviated kidney fibrosis and mesangial expansion as compared to vehicle treatment. Only PDX therapy was effective at preserving the heart function and survival rate. These results show that dietary LCω3FA intake can prevent ESRD and cardiac dysfunction in LRKOB100/IGFII diabetic mice. Our data

Abbreviations: 17-HDHA, 17-hydroxy-DHA; BUN, blood urea nitrogen; BW, body weight; CD11b, cluster of differentiation molecule 11b; colI, collagen type I; colIV, collagen type IV; CVD, cardiovascular disease; DHA, docosahexaenoic acid; DN, diabetic nephropathy; DPA, docosapentaenoic acid; DSS, dextran sodium sulfate; EPA, eicosapentaenoic acid; ESRD, end-stage renal disease; eWAT, epididymal WAT; FN1, fibronectin-1; FO, fish oil; GBM, glomerular basement membrane; GFR, glomerular filtration rate; GH, growth hormone; HDL, high-density lipoprotein; HFHS, high-fat/high-sucrose; IGFII, insulin-like growth factor II; IL, interleukin; iNOS, inducible nitric oxide synthase; IVCT, isovolumic contraction time; IVRT, isovolumic relaxation time; iWAT, inguinal WAT; KIM-1, kidney injury molecule-1; LCω3FA, long chain omega-3 polyunsaturated fatty acids; LDL, low-density lipoprotein; LDLr, LDL receptor; LF, low-fat; LOX, lipoxygenase; LRKOB100/IGFII, LDLr$^{-/-}$/ApoB$^{100/100}$ mice with transgenic overexpression of IGFII in pancreatic β-cells; LV, left ventricle; LVEF, left ventricular ejection; LVET, left ventricular ejection time; LVH, left ventricular hypertrophy; LVIDd, LV interior diameter at end-diastole; LVOT, LV outflow-tract diameter; LVRWT, LV relative wall thickness; LY6G, lymphocyte antigen 6 complex locus G6D; MPI, myocardial performance index; NLRP3, nucleotide-binding oligomerization domain like receptor containing pyrin domain 3; oGTT, oral glucose tolerance test; PAS, periodic acid Schiff stain; PD1, protectin D1; PDHc, pyruvate dehydrogenase complex; PDX, protectin DX; RAAS, renin–angiotensin–aldosterone system; RAS, renin-angiotensin system; rpWAT, retroperitoneal WAT; SGLT2, sodium–glucose co-transporter 2; SPM, specialized pro-resolving mediators; TG, triglycerides; TGF-β, transforming growth factor β; TNF-α, tumor necrosis factor-α; WAT, white adipose tissue.

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further reveals that PDX can protect against renal failure and cardiac dysfunction, offering a potential new therapeutic strategy against ESRD.

KEYWORDS

CVD, diabetic nephropathy, end-stage renal disease, omega-3 fatty acids, protectin DX

1 | INTRODUCTION

T2D-related complications are classified as microvascular (ie, nephropathy, retinopathy) and macrovascular (ie, cardiovascular disease [CVD]) according to the affected vascular bed.[1] Nearly 10% of T2D-related deaths are due to renal failure,[2] and diabetic nephropathy (DN) is the leading cause of end-stage renal disease (ESRD).[3]

The major underlying mechanisms connecting diabetes to nephropathy are hyperglycemia and intraglomerular hypertension.[4] Sustained high glucose and blood pressure levels induce chronic activation of several pro-inflammatory mediators targeting structural and functional glomerular alterations, potentially leading to renal failure.[5] Glycemic management is accordingly the first line therapy against DN[6] along with inhibitors of the Renin-Angiotensin System (RAS).[7] However, these treatments only mitigate disease progression but rarely reverse, or stall, preexisting illness. Among patients with established ESRD the only available treatments are dialysis and/or kidney transplantation; both followed by a high risk of infection and CVD event.[8] Thus, novel therapeutic strategies are warranted to prevent DN and the progression towards ESRD.

The LCω3FA eicosapentaenoic (EPA) and docosahexaenoic (DHA) are well documented anti-inflammatory mediators in part by competing with the pro-inflammatory arachidonic acid pathway.[9] At the resolution phase of inflammation, EPA and DHA are converted into potent lipid derivatives, such as resolvins and protectins, also called specialized pro-resolving mediators (SPM).[10] Persistent unresolved inflammation is a shared feature among many chronic inflammatory disorders, including DN. Several studies have shown that LCω3FA-rich fish oil (FO) consumption alleviates kidney injury by down-regulating proteinuria progression,[11,12] inflammation,[13] and hypertension.[14] In DN, omega-3 rich diet prevented glomerulosclerosis and tubulointerstitial fibrosis in rats with streptozotocin-induced diabetes.[15] However, the efficacy of LCω3FA against DN is still controversial in humans, as recently demonstrated by de Boer et al who reported that omega-3 fatty acids supplementation for 5 years did not preserve glomerular filtration rate (GFR) in patients with T2D.[16] This might be related to the limitation of using LCω3FA and calls for investigating more specific therapeutics such as LCω3FA-derived specialized resolving mediators.

DHA is the precursor of D-series resolvins and protectins via lipoxygenase (LOX), attenuating inflammatory disorders by promoting the resolution of inflammation.[9] 17S-hydroxy DHA (17-HDHA) is a DHA metabolite and precursor of Protectin D1 (PD1) and its isomer, Protectin DX (PDX).[17] Mounting evidence corroborates the pro-resolution role of both 17-HDHA and PDX. Treatment with 17-HDHA was shown to stimulate the anti-inflammatory macrophage subtype (M2 phenotype) and reduce TNF-α and inducible nitric oxide synthase (iNOS) in macrophages, while alleviating dextran sodium sulfate (DSS)-induced colitis.[18] In a mouse model of sepsis, PDX was reported to modulate macrophage phenotype, down-regulating inflammation and increasing survival rate.[19] 17-HDHA therapy was also found to attenuate obesity-linked adipose tissue inflammation and to improve insulin sensitivity in mice.[20] Recently, our laboratory documented that PDX can exert anti-diabetic effects through improving hepatic insulin sensitivity in experimental and genetic models of obesity and insulin resistance by promoting interleukin-6 (IL-6) secretion from skeletal muscle.[21] In kidney dysfunction, D-resolvin and PD1 were shown to protect kidneys from ischemic injury, reducing leukocyte influx and fibrosis in mice.[22] Moreover, DHA and 17-HDHA abrogated glomerular sclerosis through nucleotide-binding oligomerization domain like receptor containing pyrin domain 3 (NLRP3) inflammasome inhibition.[13]

Herein, we took advantage of the severe diabetic phenotype of HFHS-fed LDLr$^{-/-}$/ApoB$^{100/100}$ mice overexpressing IGFII in pancreatic β-cells (LRKOB100/IGFII), a model that combines the genetic deletion of the low-density lipoprotein (LDL) receptor (LDLr) with the overexpression of insulin-like growth factor II (IGFII) in pancreatic β-cells, to study the impact of LCω3FA and DHA-derived resolving mediators (17-HDHA and PDX) on ESRD. We report that the consumption of FO-rich LCω3FA prevented proteinuria, glomerular sclerosis, and cardiac failure in this model. Moreover, HFHS-fed LRKOB100/IGFII mice treated with 17-HDHA had reduced podocyte foot process effacement. Only 6 weeks of PDX treatment, showed improved glomerular fibrosis and mesangial expansion, increasing survival rate, potentially through preserving cardiac function, an effect that was independent from its glucoregulatory effect.

2 | METHODS

2.1 | Animals

8-11-week-old male LDLr$^{-/-}$/ApoB$^{100/100}$ mice overexpressing IGFII in pancreatic β-cells (LRKOB100/IGFII) from our in-house colony were individually housed with food and water ad libitum, at the Quebec Heart and Lung Institute facility in a 12h regulated daylight cycle. All in vivo evaluations and non-terminal samples were performed/collected during daylight. Animals were separated into experimental groups so the average of body weight (BW) baseline or treatment, respectively, did not differ significantly between groups (Figure S1A). Body weight (BW) gain was assessed once a week and food intake three times a week.

2.1.1 | Dietary intervention

Following 2 weeks of acclimation on a normal chow diet (Teklad, Harlan), 8-11 week-old mice were fed either a low-fat (LF, n = 15) diet containing 16% of total kcal from lipids (50% corn oil and 50% lard), 70% from carbohydrates (56% starch and 14% sucrose), and 14% from proteins (Casein), a high-fat/high-sucrose (HFHS) diet containing 65% of kcal from lipids (50% corn oil and 50% lard), 20% from carbohydrates (5% starch and 15% sucrose), and 15% from proteins (Casein), or a HFHS diet in which 7.19% of kcal content of corn oil was replaced by fish oil (FO, mixture of 44% EPA and 25% DHA, VivoMega 4020 EE, n = 16) for 26 weeks (Table 1). All diets contained 0.2% cholesterol to accelerate atherosclerosis development.

2.1.2 | Therapeutic treatment regimen

After 21-22 weeks of HFHS feeding mice received daily treatments (by oral gavage) of either corn oil (Mazola® 100% pure, cholesterol free) as vehicle (CNTL, n = 18), 17-HDHA (50ng/g of BW; Cayman Chemical, n = 18) or Protectin DX (PDX, 30 ng/g of BW; Santa Cruz Biotechnology, n = 12) for 6 weeks (Figure 5A) from 8-11 AM in alternating order. The 17-HDHA dose was based on a previous study showing that 50 ng of 17-HDHA/g of BW reduced adipose tissue expression of inflammatory cytokines, increased adiponectin expression, and improved glucose tolerance in obese mice.[20] FO-fed mice were also daily treated with vehicle (120 µL of corn oil) to be comparable to CNTL group. Only a single CNTL group was applied to lower the number of mice used (for ethical reasons). The CNTL group was thus used for both preventive and therapeutic experiments. Statistical adjustments were applied for this confounder, see *statistical analysis* section for further explanations.

TABLE 1 Diet composition

kcal %	LF	CNTL	FO
Protein	14.3	14.7	14.7
Carbohydrate	70.2	19.8	19.8
Fat	15.5	65.5	65.5
Casein	14.3	14.7	14.7
Starch	56.2	4.9	4.9
Sucrose	12.5	13.3	13.3
Lard	7.6	32.7	32.7
Corn oil	7.6	32.7	25.5
LCω3FA-rich fish oil	–	–	7.2

At the end of the protocol, mice were anesthetized with isoflurane and euthanized by cardiac puncture. Epididymal, retroperitoneal, and inguinal white adipose tissues (eWAT, rpWAT, and iWAT, respectively) from kidney and heart were collected. Animals from the same batch were euthanized on the same day from 8 AM to 5 PM immediately after echocardiography assessment, in alternating order. All animal protocols were approved by the Animal Care Committee of Laval University.

2.2 | Analytical methods

Lipid profile and blood urea nitrogen (BUN) were measured in plasma from 6 hr-fasted mice at week 18 and BUN was also measured at week 26. Blood was drawn at 8-9 AM in alternating order into EDTA-containing tubes and centrifuged to isolate plasma that was stored at −80°C for further analysis. Standard colorimetric kits were used for triglycerides (TG; TR22421; Thermo Scientific), cholesterol (CH200; Randox), high-density lipoprotein (HDL; CH2652; Randox) and low-density lipoprotein (LDL; CH2657; Randox). BUN was measured using biochromatic rate technique (BUN Flex reagent cartridge) on the Dimension Vista® System. At week 25, mice were 12-hr fasted and submitted to an oral glucose tolerance test (oGTT) at 8 AM Insulin concentration was measured in plasma collected during the oGTT with the use of the Ultrasensitive mouse ELISA (EMD Millipore). Urine was collected at week 26 in anesthetized mice prior to echocardiography assessment. Briefly, gentle pressure was applied to the transabdominal area, favoring the urine out of the bladder. Urinary creatinine and albumin concentration were estimated by a colorimetry assay (The Creatinine Companion; Exocell) and ELISA (Albuwell M; Exocell), respectively.

2.3 | Histopathology

At sacrifice, the inferior half of the right kidney was isolated and fixed in 4% paraformaldehyde/PBS and then transferred

to 70% ethanol for mesangial cell expansion and immunohistochemistry analysis. Tissues were embedded with paraffin and sectioned (4 μm).

2.3.1 | Mesangium expansion and glomerular fibrosis

Quantitative mesangial cell expansion was estimated in 4 μm sections stained with periodic acid Schiff stain (PAS) and hematoxylin-eosin. Briefly, the relative number of pixels of the mesangium was divided by the total area of each glomerulus by using a binary threshold on Image J (V. 1.51j8, NIH, USA).[23] Renal fibrosis was assessed on Masson's Trichrome stained slides. The relative number of pixels of the fibrotic glomerulus was divided by the total area of each glomerulus by using a binary threshold on Image J (V. 1.51j8, NIH, USA) as per mesangium expansion quantification.

2.4 | Transmission electron microscopy

2.4.1 | Foot process effacement

Renal cortex was isolated from the left kidney and a 2 mm square was kept under 25% glutaraldehyde solution for later electronic microscopy transmission and scanning for podocyte foot process effacement evaluation as previously described.[23]

2.5 | Immunohistochemistry

Immunohistochemistry of kidney sections was performed with the ABC Kit (Vector Laboratories). Sections were blocked using an avidin/biotin blocking Kit (Vector Laboratories, SP-2001). Primary antibodies against transforming growth factor β (TGF-β; 1:200 dilution, Santa Cruz, sc-146) and collagen type IV (colIV; 1:2500 dilution, Novus biological, NB110-5998) were used for immunohistochemistry analyses. Positive staining was obtained by incubating sections in 3,39-diaminobenzidine solution (DAB kit, Vector Laboratories, sk-4100) following by a counterstaining of the nucleus using Gill's Hematoxylin (Vector Laboratories, H-3401).

2.6 | Lipidomic analysis

Heart and plasma lipid mediators were assessed using liquid chromatography associated with tandem mass spectrometry (LC-MS/MS) and an electrospray interface. The LC-MS/MS was performed using an Alliance 2690 HPLC apparatus (Waters) and an API3200 mass spectrometer (Applied Biosystems).

2.7 | Magnetic resonance imaging

Body composition was estimated at weeks 0, 18, and 26 between 8 and 11 AM in alternating order by magnetic resonance imaging using the Bruker's Minispec LF90II (Bruker Optics, Germany). Fat and lean mass were expressed as the average of three consecutives measurements per mouse.

2.8 | Echocardiography

Transthoracic echocardiography was performed at weeks 18 and 26 between 8 AM to 5 PM in alternating order under isoflurane anesthesia with the L15-7io (5-12 Megahertz) and S12-4 (4-12 Megahertz) probes connected to a Philips HD11XE ultrasound system (Philips Healthcare Ultrasound, The Netherlands) as previously described.[24] In short, left ventricular (LV) dimensions: LV interior diameter at end-diastole (LVIDd), LV relative wall thickness (LVRWT) and LV outflow-tract diameter (LVOT) were acquired in M-mode imaging of parasternal short-axis view. Left ventricular ejection (LVEF) and LV mass calculations were based on LV dimentions.[24] Transmitral (A and E wave) and LVOT outflow velocity were accessed by pulsed-wave Doppler. While mitral annulus motion velocity (E´ wave) was measured using tissue imaging Doppler. Stroke volume was based on LVOT outflow and cardiac output was estimated by the product of stroke volume and heart rate. Myocardial performance index (MPI) was calculated by the ratio between the sum of isovolumic contraction time (IVCT) and isovolumic relaxation time (IVRT) by the left ventricular ejection time (LVET).

2.9 | Real-time polymerase chain reaction

Total RNA was isolated from homogenized kidney by using a GeneJETRNA purification kit (Thermofisher, Canada). One microgram of RNA was reverse transcribed using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Canada) and Quantitative real-time PCR (qPCR) was performed with Quantitec SYBR Green PCR kit (Qiagen, Canada). GAPDH mRNA expression was used for normalization.

2.10 | Statistical analysis

Data are expressed as mean ± SEM. Statistical analysis from the dietary FO-replacement was performed using

Student's *t*-test (GraphPad, USA) *versus* CNTL. One-way ANOVA was performed for the gavage intervention strategy comparisons *versus* CNTL and a Dunnett's multiple comparisons test was added (GraphPad, USA). Body weight gain, oGTT, insulinemia during oGTT, echocardiography data and BUN were statistically compared using two-way repeated measures ANOVA with a Bonferroni post hoc test (Sigmaplot, USA) Differences on survival rate were measured by Logrank (Mantel-Cox) test. LF-fed mice were not included in any statistical comparisons providing only a reference of a low-fat/low-sucrose feeding. Since the CNTL group was used as the reference group for both strategies, *P* values from FO and lipid mediator tests were multiplied by 2 and 1.5, respectively, in order to correct the *P* value to the accurate number of comparisons (manual Bonferroni post hoc test). All results were considered statistically significant at $P < .05$.

3 | RESULTS

3.1 | Dietary fish oil prevents kidney dysfunction and death while preserving insulin secretion

Mice overexpressing IGFII in pancreatic β-cells are known to develop T2D, cardiac dysfunction and aortic stenosis which is further accelerated when combined with an obesogenic diet.[24,25] Here, we further report that chronic HFHS feeding also lead to severe kidney injury in this model.

LRKOB100/IGFII mice were fed with LF, HFHS (CNTL group) or the FO diet for 26 weeks (Figure 1A). The substitution of ~7% of dietary corn oil for FO led to increased circulating levels of EPA, DHA and docosapentaenoic acid (DPA) (Figure 1B). FO-feeding similarly augmented both DHA-derived mediators, 17-HDHA and PDX (Figure 1C) as compared to CNTL mice, although only PDX was statistically significant (Figure 1C). Starting at week 17, HFHS-fed mice exhibited a dramatic reduction in BW (Figure 1D) and survival rate (Figure 1E). FO feeding preserved BW and survival rate (Figure 1D,E). Furthermore, dietary FO prevented kidney dysfunction, exemplified by reduced kidney weight (Table 2), albumin-to-creatinine ratio (Table 2), and BUN (Figure 1F) compared to CNTL group.

The prevention of kidney dysfunction could not be explained by improved glucose handling as compared to vehicle treated animals (Figure S1C) despite partial restoration of glucose-induced insulin response in the former group (Figure S1D). The relatively improved glucose tolerance in the CTLN group is likely related to the nearly 25% weight loss as compared to FO-fed animals (insert Figure 1D).

3.2 | Dietary fish oil prevents kidney inflammation

Unresolved inflammation is a shared feature among chronic metabolic disorders, including DN. Dietary FO replacement down-regulated renal inflammation in comparison with CNTL group, as demonstrated by reduced tumor necrosis factor-α (TNF-α), interleukin-1β (IL-1β) and iNOS gene expression in the kidney cortex (Figure 2A). FO-fed mice tended ($P = .07$) to have reduced leucocyte infiltration in the glomerulus (Figure 2B), as revealed by reduced immunofluorescent intensity of lymphocyte antigen 6 complex locus G6D (LY6G, Figure 2C). We also observed reduced monocyte infiltration, as seen by cluster of differentiation molecule 11b (CD11b, Figure 2D) per glomerulus, although this was not statistically significant given the high level of variation of this marker.

Podocyte cells are important components of glomerular filtration barrier providing structural and functional support to glomerular filtration.[4] Podocyte foot process effacement and cell death are processes that contributes to glomerular dysfunction and proteinuria in DN.[5] In parallel with augmented inflammation, CNTL group also showed enhanced podocyte foot process effacement as revealed by high podocyte foot process width, which tended to be reduced by FO treatment (Figure 2E,F).

3.3 | Dietary fish oil prevents mesangial expansion and glomerular fibrosis

Glomerular mesangial expansion is a typical pathohistological alteration observed in DN that strongly correlates with GFR.[26] LRKOB100/IGFII mice under long-term HFHS feeding developed severe mesangial expansion while FO consumption was able to inhibit such structural remodeling (Figure 3A,B).

Inflammatory cytokines can activate fibroblasts, which contribute to renal fibrosis,[27(p88)] a central process of ESRD development. Stained renal cortex with Masson's Trichrome technique revealed a severe fibrotic condition in the CNTL group, noted by a wide-spread and intense blue (collagen-related) staining (Figure 3C) and glomerular quantification (Figure 3D). The effect was not restricted to the glomerulus, as renal tubules were also largely stained and hypertrophic (Figure 3C). This phenotype was fully prevented by FO treatment (Figure 3C,D).

We next evaluated whether collagen type IV was involved in such severe fibrosis. Collagen type IV is a major basement membrane protein and its upregulated in several glomerular diseases.[28-30] Dietary FO reduced the mean of colIV deposition as compared to CNTL group, although such modulation was not statistically significant (Figure 3E,F).

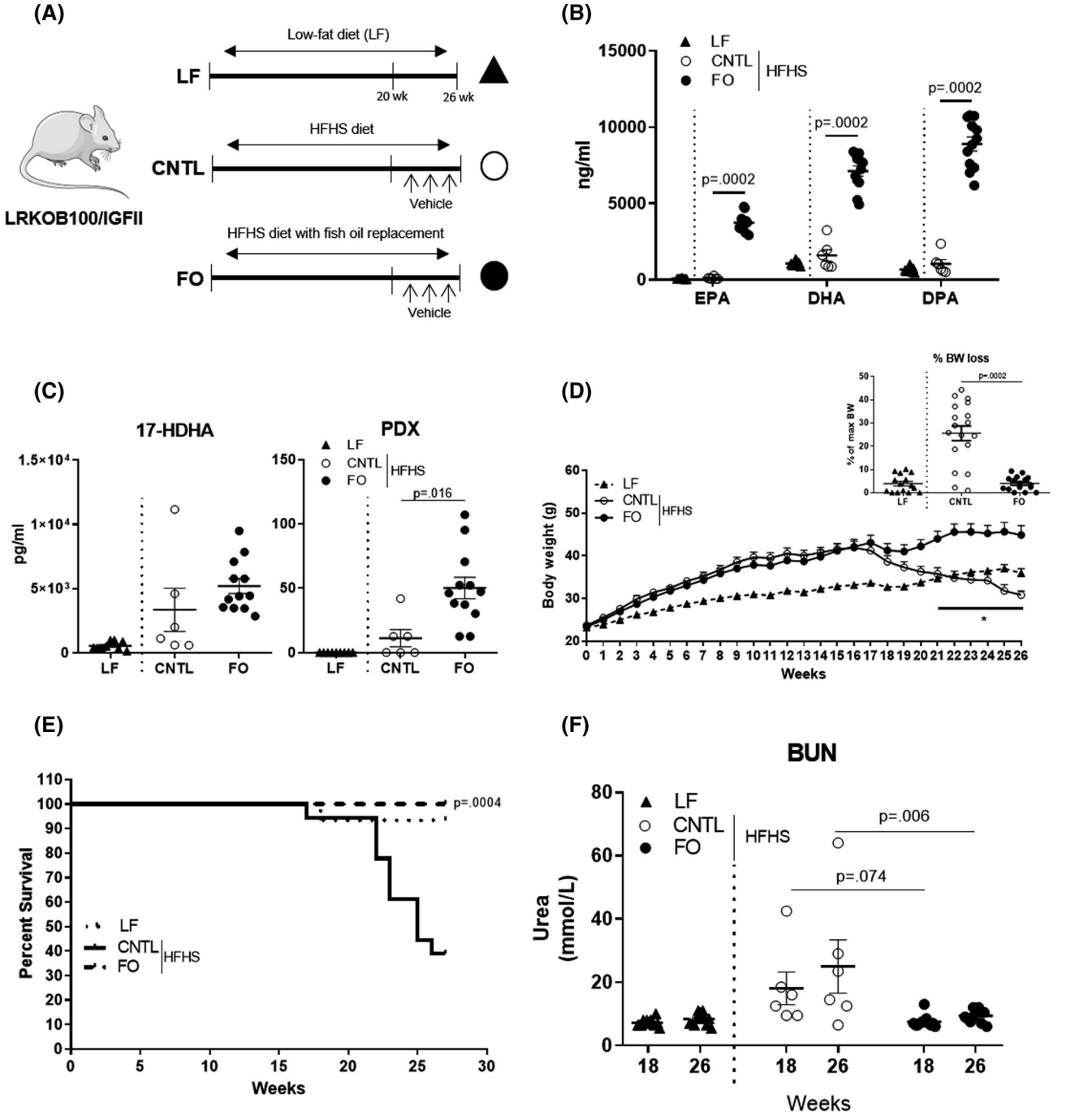

FIGURE 1 Dietary fish oil prevents kidney dysfunction and death. A, Prevention strategy overview. FO increased circulating levels of (B) EPA, DHA and DPA as compared to CNTL, measured in fasted plasma collected at week 26 (CNTL n = 6/FO n = 12). C, PDX, but not 17-HDHA, was also enhanced is plasma of mice fed with FO diet *versus* CNTL (CNTL n = 6/FO n = 12). FO-feeding prevented ESRD development as shown by continued (D) body weight (BW) gain (CNTL n = 18/FO n = 16), preserved (E) percent survival (CNTL n = 18/FO n = 16) and reduced (F) blood urea nitrogen (BUN) as compared to CNTL mice (CNTL n = 6/FO n = 12). Lipid mediators, BW gain and BUN are expressed as mean ± SEM, while survival rate is expressed as percentage of cases. Statistical differences on BW and BUN were calculated using two-way repeated measures ANOVA with a Bonferroni post hoc test *versus* CNTL. Differences on survival rate were measured by Longrak (Mante-Cox) test *versus* CNTL. Data for LF-fed mice are shown as a reference and were not included in statistical comparisons (indicated by the hatched line)

Concomitant with severe fibrosis, HFHS-fed/vehicle-treated mice presented augmented gene expression of pro-fibrotic factors, such as colIV, TGF-β, collagen type I (colI) and fibronectin-1 (FN1) in renal cortex, compared to FO-fed mice (Figure 3G). Kidney injury molecule-1 (KIM-1) is a type I trans membrane protein expressed in proximal epithelial cells after renal injury.[31] FO-fed mice exhibited a striking 60-fold lower KIM-1 expression in the

TABLE 2 Effects of fish oil replacement and 17-HDHA and PDX treatment on metabolic data of LRKOB100/IGFII mice

	LF	FO	CNTL	17-HDHA	PDX
Kidney weight. g (week 26)	0.269 ± 0.006	0.304 ± 0.011**	0.415 ± 0.037	0.366 ± 0.017	0.376 ± 0.021
Albumin. µg/dL (week 26)	4.24 ± 0.496	6.97 ± 1.488	6.726 ± 0.410	4.455 ± 0.625	6.981 ± 0.410
Creatinine. mg/dL (week 26)	71.865 ± 10.70	79.347 ± 13.07**	19.123 ± 1.664	30.175 ± 13.997	28.045 ± 3.281
Albumin/creatinine. µg/mg (week 26)	66.13 ± 7.112	97.61 ± 18.40***	358.0 ± 25.44	244.8 ± 69.85	268.2 ± 38.19
Fasting plasma glucose. mmol/L (week 25)	10.512 ± 0.285	11.00 ± 0.715	9.525 ± 1.295	10.133 ± 0.894	10.062 ± 0.766
Fasting plasma triglycerides. mmol/L (week 18)	15.41 ± 1.892	28.99 ± 5.941	24.37 ± 4.120	20.72 ± 2.389	15.55 ± 3.178
Fasting plasma cholesterol. mmol/L (week18)	14.75 ± 0.623	18.35 ± 1.610*	11.40 ± 0.828	12.08 ± 1.038	11.33 ± 0.611
Fasting plasma LDL. mmol/L (week18)	8.396 ± 0.409	9.315 ± 0.734**	5.65 ± 0.501	5.924 ± 0.616	5.478 ± 0.279
Fasting plasma HDL. mmol/L (week18)	0.537 ± 0.064	0.695 ± 0.076	1.002 ± 0.145	0.905 ± 0.068	1.042 ± 0.079
Visceral white adipose tissue. g (week 26)	2.210 ± 0.146	3.628 ± 0.195***	1.769 ± 0.220	1.613 ± 0.428	1.649 ± 0.278
Subcutaneous white adipose tissue. g (week 26)	0.917 ± 0.073	2.149 ± 0.256*	1.103 ± 0.108	1.078 ± 0.280	0.907 ± 0.178

renal cortex as compared to vehicle-treated CNTL mice (Figure 3G).

3.4 | Dietary FO prevents lean mass loss and cardiac failure

Muscle wasting is a common feature of ESRD.[32,33] Muscle mass loss is associated with increased mortality in ESRD patients.[34] FO feeding resulted in higher lean mass as compared to CNTL mice already at week 18, a time at which both groups still shared a similar fat mass (Figure 4A,B). Moreover, the urinary creatinine levels were preserved among the FO-fed, while drastically low in CNTL mice (Table 2).

Patients with kidney failure are susceptible to CVD comorbidities such as coronary artery disease, sudden death/cardiac arrhythmias, myocardial infarction, stroke, and congestive heart failure.[35] Although, in humans, left ventricular hypertrophy (LVH) is the most frequent cardiac alteration in ESRD,[36] no statistical differences on absolute LV mass values were noted from week 18 to 26 in CNTL mice (Figure 4C). Instead, ESRD in HFHS-fed/vehicle-treated mice were associated with reduced heart rate from week 18 to 26 (Figure 4D) paralleling prolonged diastolic (Figure 4E) and systolic time (Figure 4F) that resulted in higher MPI (Figure 4G). All of which was prevented by FO feeding. Indeed, ESRD leads to cardiac fibrosis and LV systolic and diastolic dysfunction, potentially contributing to sudden death.[37] Thus, these data suggest a FO-related prevention against cardiac failure potentially by modulating heart rate and global systolic and diastolic ventricular function, which could potentially be related to a prior volume overload. Furthermore, in FO-fed mice, cardiac output was augmented from week 18 to 26 (Figure 4H), resulting in higher cardiac output at week 26 as compared to CNTL (Figure 4H). Paralleling the FO-related cardiac output effect, stroke volume also tended ($P = .05$) to be increased overtime (Figure 4I).

Increased plasma volume may result in high cardiac filling pressures, which can lead to heart failure with preserved ejection fraction.[38] Likewise, CNTL mice had heart failure with preserved ejection fraction. FO feeding, although preventing heart rate and global systolic and diastolic impairments, had lower LVEF at week 26 as compared to CNTL (Figure 4J). This might be related to the higher weight gain observed in the FO group, as obesity often correlates with LVEF depression.[24]

3.5 | PDX treatment preserves survival rate while decreasing mesangial expansion and fibrosis

LRKOB100/IGFII mice were daily gavaged with either 17-HDHA or PDX during the final 6 weeks of the protocol; CNTL group received vehicle (Figure 5A). Until the end of the protocol, survival rate in PDX-treated mice remained at 83% versus only 39% for CNTL group (Figure 5B). No statistical difference for glucose homeostasis was noted among groups, as shown by both glycemia (Figure S1E) and insulinemia during oGTT (Figure S1F). The DHA-derived lipid mediators did not affect inflammatory markers in the kidney cortex at the transcriptional level (Figure 5C). However, PDX alleviated structural glomerular damage as revealed by reduced mesangial expansion (Figure 5D,E). On the other hand, 17-HDHA treatment reduced podocyte foot process effacement as compared to CNTL (Figure 5F,G). Despite no statistical difference on albumin to creatinine ratio (Table 2) and on leucocyte infiltration within the glomerulus (Figure S2C,E), PDX treatment reduced glomerular fibrosis to half of that observed in vehicle-treated CNTL mice (Figure 6A,B). Intriguingly, such modulation on renal fibrosis may only partly be linked to lower colIV deposition, as the stained mesangium area percent was not

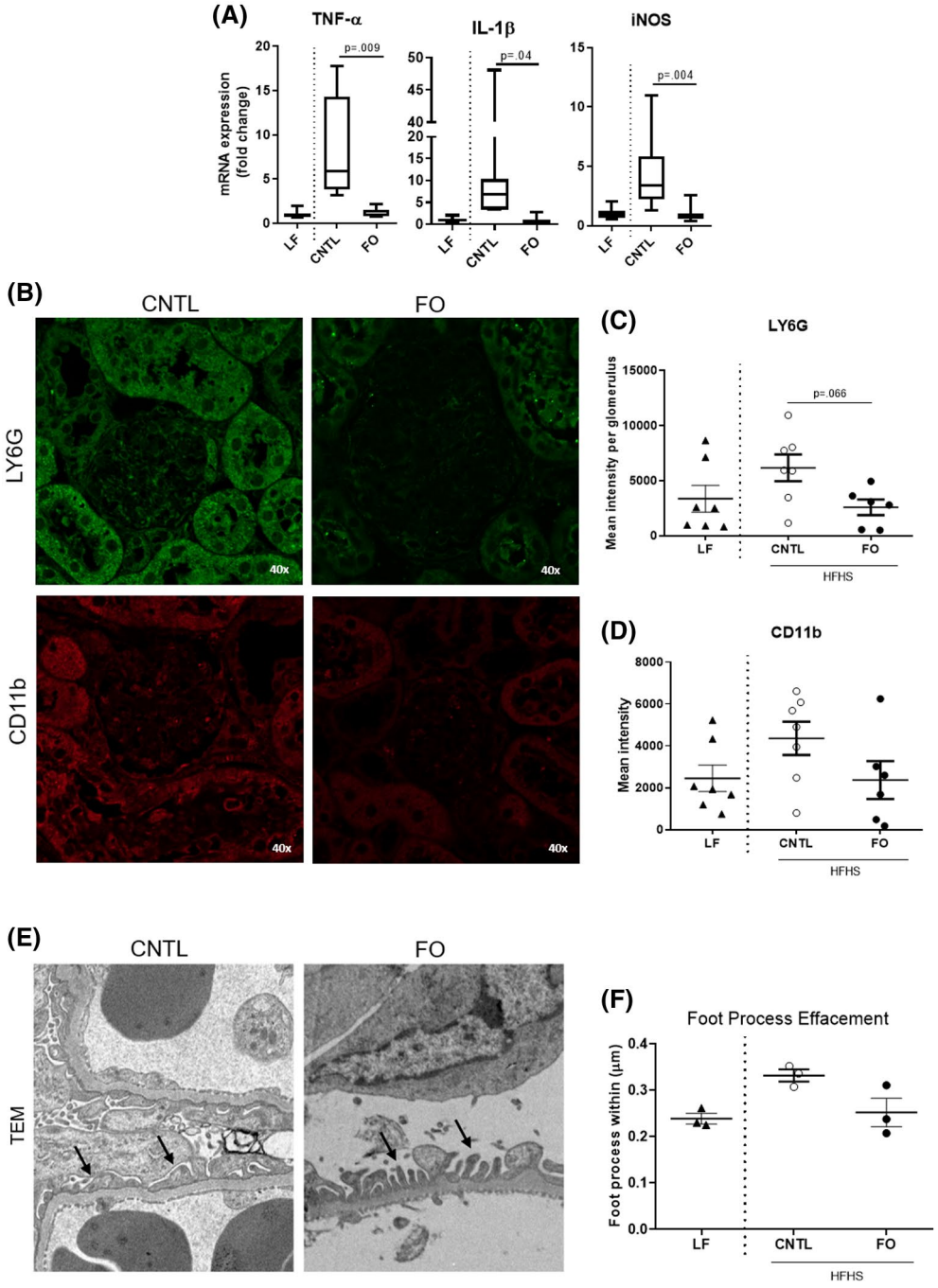

FIGURE 2 Dietary fish oil replacement prevents kidney inflammation. FO-feeding prevented increased (A) gene expression of inflammatory markers (CNTL n = 7/FO n = 8) as well as leucocytes and monocyte infiltration within the glomerulus as revealed by reduced immunofluorescent (B) intensity and a trend for quantification of (C) lymphocyte antigen 6 complex locus G6D (LY6G), while (D) cluster of differentiation molecule 11B (CD11b) was not statistically different between groups (CNTL n = 7/FO n = 6). FO-replacement did not statistically modulate (E, F) podocyte foot process effacement as compared to CNTL mice (CNTL n = 3/FO n = 3). Data on gene expression of inflammatory markers are expressed as box plot diagram. Mean intensity per glomerulus quantification and foot process within are expressed by mean ± SEM. All statistical analyses were performed using Student's *t*-test of FO-fed *versus* CNTL. Data for LF-fed mice are shown as a reference and were not included in statistical comparisons (indicated by the hatched line)

statistically reduced by PDX (Figure 6C,D). 17-HDHA and PDX did not affect glomerular fibrosis at the transcriptional levels as determined by colI, colIV and TGF-β gene expression (Figure 5C).

3.6 | PDX protects against cardiac failure

CVD is the leading cause of death among ESRD patients.[39] We thus evaluated the potential therapeutic effect

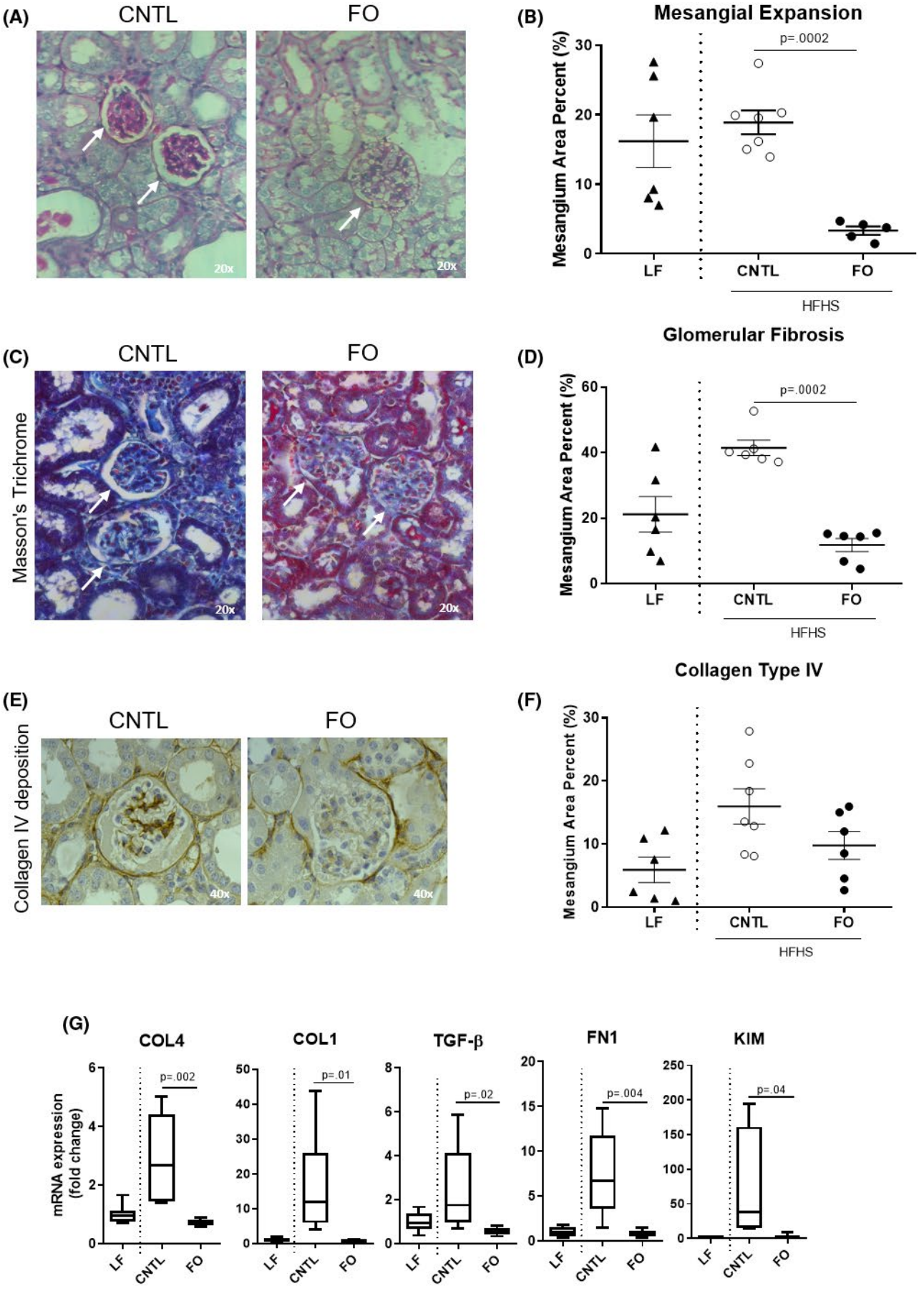
(A)
CNTL
FO
20x
20x
(B)
Mesangial Expansion
Mesangium Area Percent (%)
p=.0002
LF
CNTL
FO
HFHS
(C)
CNTL
FO
Masson's Trichrome
20x
20x
(D)
Glomerular Fibrosis
Mesangium Area Percent (%)
p=.0002
LF
CNTL
FO
HFHS
(E)
CNTL
FO
Collagen IV deposition
40x
40x
(F)
Collagen Type IV
Mesangium Area Percent (%)
LF
CNTL
FO
HFHS
(G)
COL4
COL1
TGF-β
FN1
KIM
mRNA expression (fold change)
p=.002
p=.01
p=.02
p=.004
p=.04
LF
CNTL
FO

FIGURE 3 Dietary fish oil replacement prevents mesangial expansion and glomerular fibrosis. FO-fed mice showed reduced (A) glomerular mesangial expansion as noted by staining and (B) quantification as well as (C, D) glomerular fibrosis. Although reducing the mean, no statistical differences were noted for the effect of FO on (E) type IV collagen (colIV) deposition within the glomerulus expressed as (F) mesangium area percent (CNTL n = 7/FO n = 5-6). It also prevented increased (G) gene expression of fibrotic markers in kidney cortex (CNTL n = 7/FO n = 8). Data are expressed by mean ± SEM. All statistical analyses were performed using Student's *t*-test of FO-fed *versus* CNTL. Data for LF-fed mice are shown as a reference and were not included in statistical comparisons (indicated by the hatched line)

of both 17-HDHA and PDX against cardiac dysfunction. Echocardiography was performed at week 18, prior to the initiation of the therapeutic treatment regimen (at week 20), and 6 weeks after treatment initiation (at week 26). While mice receiving PDX treatment from week 20 had lower left ventricular ejection fraction at week 18 as compared to their time-matched CNTL counterparts, it still augmented ejection fraction at week 26 *versus* week 18 (Figure 6E). From week 18 to 26, HFHS-fed vehicle treated CNTL mice had reduced heart rate, an effect that was prevented by PDX, but not by 17-HDHA treatment (Figure 6F). 17-HDHA treated mice had a trend towards increasing stroke volume ($P = .08$, Figure 6G). Moreover, PDX treatment increased cardiac output, resulting in higher values at week 26 as compared to CNTL group (Figure 6H). Global systolic and diastolic function, as expressed by MPI was increased between 18 and 26 weeks in vehicle-treated CNTL mice (Figure 6I). Both 17-HDHA and PDX treatments did not significantly impact MPI (Figure 6I). Yet, at week 26, only mice treated with PDX showed a strong trend ($P = .06$) for lower MPI levels as compared to CNTL (Figure 6I).

4 | DISCUSSION

LCω3FA provide a large range of physiological benefits that includes insulin sensitization,[40] resolution of inflammation[9] and anti-hypertensive modulation.[41] Much less is known about their potential benefits for DN. While LCω3FA were reported to reduce proteinuria,[42] the impact of fish oil consumption on renal function and ESRD progression remains contradictory.[43] To the best of our knowledge, we show for the first time that long-term fish oil replacement prevents progression to ESRD and cardiovascular dysfunction in a murine model of T2D. We also provide evidence for the potential therapeutic benefits of both DHA-derived resolution mediators; 17-HDHA and PDX on ESRD and associated cardiac dysfunction.

The combination of a long-term obesogenic diet with the diabetic-prone genotype of LRKOB100/IGFII mice lead to ESRD progression. This was not seen in LDLrKOB100 mice (data not shown), suggesting that the overexpression of IGF-II factor in pancreatic β cells, which accelerates their failure and generates a more severe diabetic phenotype[25] is crucial to the development of DN in this model. Unexpectedly, at week 17 some HFHS-fed LRKOB100/IGFII mice started to lose weight and die. *Post-mortem* autopsies revealed abnormally large and yellowish kidney. Perceiving the potential effect on survival rate of dietary FO replacement and PDX treatment, we elected to undertake a complete kidney profile assessment. Renal dysfunction was already present at week 18 as revealed by higher BUN in CNTL mice compared to LF reference. In agreement, body weight loss and death were triggered at the same time point.

We believe that IGF-II overexpression was directly involved in this DN phenotype of LRKOB100/IGFII. Indeed, it has been reported that mouse mutant embryos overexpressing IGF-II exhibited excessive IGF-II causing somatic overgrowth, visceromegaly (including the kidney) and cardiac defects.[44] Furthermore, a transgenic mice model which IGF II overexpression is driven by the major urinary protein promoter (highly expressed in the liver and preputial gland) showed 5%-8% reduction in lean mass and 44 to 77% of fat mass.[45] The growth hormone (GH)/-IGF-I axis is important for kidney size and function and regulates kidney IGFII expression.[46-48] Overexpression of IGF-II led to disproportionately enlarged kidneys relative to body weight.[49] It is therefore possible that IGF-II overexpression in extra-pancreatic organs contributed to accelerate kidney dysfunction in these mice.

We found that neither the dietary replacement of nearly 7% of kcal of fat with fish oil for 26 weeks, nor the treatment with DHA-derived mediators improved glucose homeostasis as compared to CNTL. However, FO-feeding seemed to preserve insulin secretion as the only group that displayed the expected insulinemic peak following hyperglycemic challenge, which is typical from the biphasic glucose-stimulated release of insulin from pancreatic islets.[50] A similar effect was reported in sucrose-fed rats, in which FO replacement was also followed by a normalization of insulin secretion pattern, that was suggested to be due to the upregulation of pyruvate dehydrogenase complex (PDHc) activity,[51] since PDHc converts pyruvate to acetyl-CoA increasing glucose oxidation and insulin secretion.[52]

Chronic unresolved inflammation is a shared feature among diabetes-related co-morbidities. Persisted inflammation results in kidney damage, such as glomerular basement membrane (GBM) thickening and disruption.[5,53] Podocytes are epithelial cells that together with the GBM and the fenestrated endothelium composes the glomerular capillary[54] and modulates glomerular filtration trough foot projections that associates with capillary vessels to form filtration slits.[55] In DN, podocyte foot process undergo effacement while leading

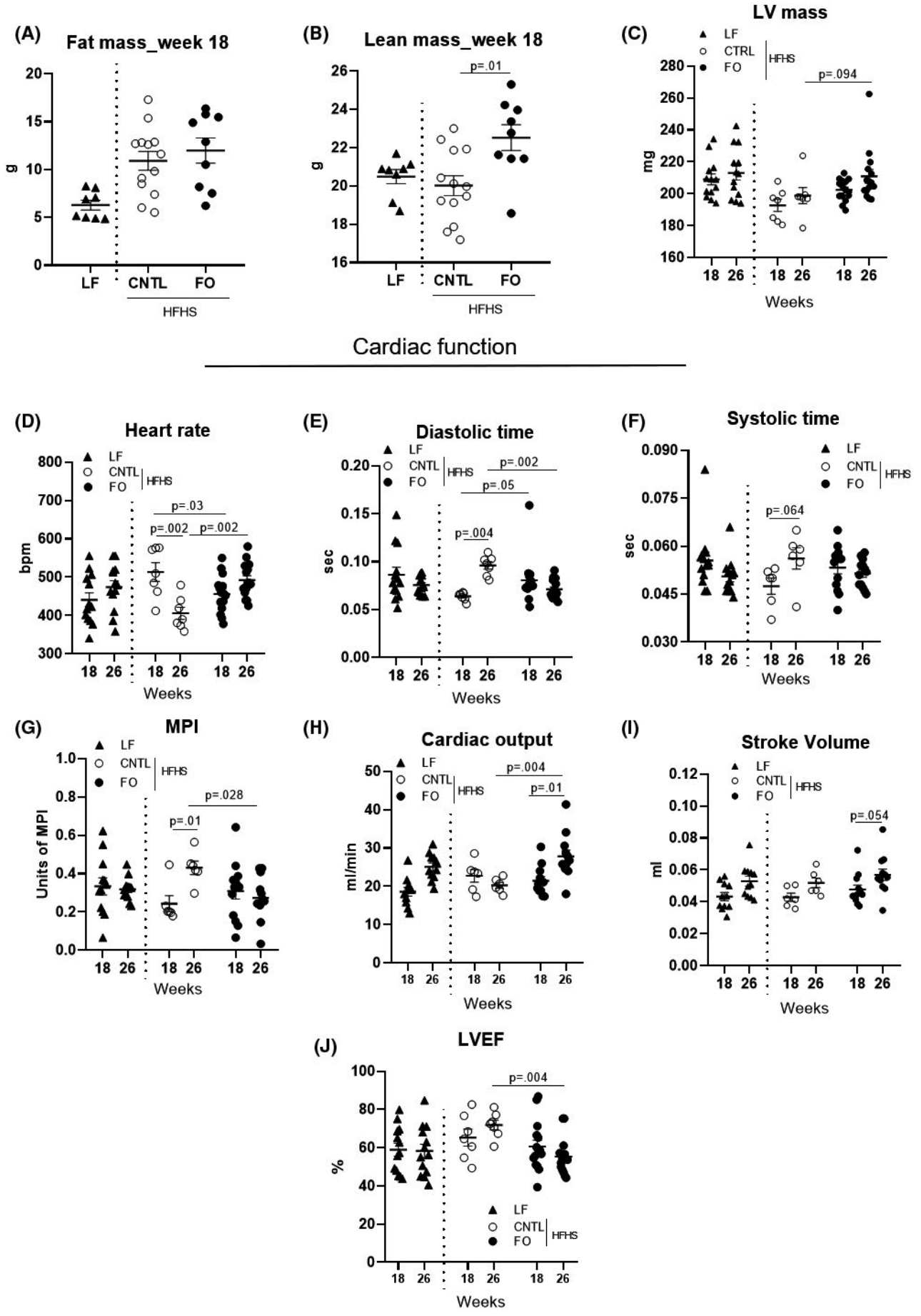
(A)
Fat mass_week 18
g
LF
CNTL
FO
HFHS
(B)
Lean mass_week 18
p=.01
g
LF
CNTL
FO
HFHS
(C)
LV mass
LF
CTRL
FO
HFHS
p=.094
mg
18 26 18 26 18 26
Weeks
Cardiac function
(D)
Heart rate
LF
CNTL
FO
HFHS
p=.03
p=.002
p=.002
bpm
18 26 18 26 18 26
Weeks
(E)
Diastolic time
LF
CNTL
FO
HFHS
p=.002
p=.05
p=.004
sec
18 26 18 26 18 26
Weeks
(F)
Systolic time
LF
CNTL
FO
HFHS
p=.064
sec
18 26 18 26 18 26
Weeks
(G)
MPI
LF
CNTL
FO
HFHS
p=.028
p=.01
Units of MPI
18 26 18 26 18 26
Weeks
(H)
Cardiac output
LF
CNTL
FO
HFHS
p=.004
p=.01
ml/min
18 26 18 26 18 26
Weeks
(I)
Stroke Volume
LF
CNTL
FO
HFHS
p=.054
ml
18 26 18 26 18 26
Weeks
(J)
LVEF
p=.004
%
LF
CNTL
FO
HFHS
18 26 18 26 18 26
Weeks

FIGURE 4 Dietary fish oil replacement increases lean mass and prevents cardiac failure. (A) fat and (B) lean mass were estimated at week 18 by magnetic resonance (CNTL n = 13/FO n = 9). Dietary modulation of cardiac function was measured by echocardiography at weeks 18 and 26. Despite similar (A) fat mass, FO-fed mice showed higher (B) lean mass, while (C) left ventricular (LV) mass was similar as compared to CNTL. Echocardiography further revealed that FO- feeding prevented a drop on (D) heart rate, as well as the drastic increase in (E) diastolic time, (F) systolic time and (G) myocardial performance index (MPI). FO-fed had also higher (H) cardiac output and a trend for higher (I) stroke volume, while (J) ejection fraction (LVEF) was similar between groups (CNTL n = 6-7/FO n = 13-15). Data are expressed as mean ± SEM. Dietary effects on fat and lean mass at week 18 were calculated by Student's *t*-test *versus* CNTL. Echocardiography data were analyzed by two-way repeated measures ANOVA with a with a Bonferroni post hoc test *versus* CNTL. Data for LF-fed mice are shown as a reference and were not included in statistical comparisons (indicated by the hatched line)

to a leaky glomerular filtration barrier that favorizes proteinuria.[56] FO-feeding diminished glomerular inflammation, however no statistical differences were noted for the podocyte foot process effacement. Contrasting, 17-HDHA treatment decreased podocyte foot process as compared to CNTL mice without modulating the inflammatory status. Furthermore, FO feeding prevented, and PDX treatment alleviated mesangial matrix expansion, a feature of DN that best correlates with declined GFR.[57]

Omega-3 fatty acids consumption was previously shown to down-regulate proteinuria in chronic glomerular diseases.[42,58,59] In line with this, long-term FO-feeding prevented the increased albumin-to-creatinine ratio and accumulation of waist products in circulation, although a timed collection of urine in metabolic cages would be needed to confirm the impact of FO on proteinuria. The benefits of FO consumption on renal function and structure prevented ESRD establishment, as revealed by preserved survival rate and BW. Previously, omega-3 consumption was shown to increase survival rate after prolonged ischemic renal injury as compared to omega-6 feeding, which was associated with decreased leukocyte infiltration and increased PD1 accumulation in kidney.[60]

The preventive nature of FO consumption against ESRD was further confirmed by a decrease in the fibrotic status of both the glomerulus and tubules, which was associated with reduced expression of pro-fibrotic factors. Despite lower colIV gene expression in kidney cortex, FO did not statistically modulate its deposition within the glomerulus, indicating that colIV might not be the main collagen type involved in FO actions. Considering that colI expression was more than 3 times higher than colIV in CNTL mice, and that FO kept expression of both genes at similar levels, it is likely that colI is the main collagen type modulating the FO effect in glomerular fibrosis. Interestingly, the FO-fed group had a similar if not better glomerular profile (ie, mesangial expansion and glomerular fibrosis) to that of the reference LF-fed group suggesting that LCω3FA consumption is a very effective approach to prevent diabetic kidney failure.

PDX therapy was shown to down-regulate glomerular fibrosis and increase animal survival rate without normalizing BW or proteinuria. In contrast to the prevention strategy with FO-replacement, PDX was unable to down-regulate some of the early factors associated with DN (ie, inflammation and transcriptional pro-fibrotic activation), but it rather modulated structural damage and advanced injury such as severe fibrosis. Although DHA-derived mediators could not completely reverse ESRD in this short-term therapeutic protocol, our data indicate that PDX slowed down the glomerular sclerosis progression, while 17-HDHA had a specific effect in podocyte foot process effacement. Whether these effects of PDX and 17-HDHA can be extrapolated to human ESRD remains unclear. Indeed, one limitation of this study is that key differences exist between the DN pathogenesis of human and murine species, in particular the severity of fibrosis or sclerotic changes of glomeruli.[61] Thus, it remains to be shown that treatment with FO-derived resolution mediators can offer a new therapeutic strategy against human ESRD.

Nearly 39% of dialysis patients' deaths, in the US, are due to CVD.[62] ESRD parallels the development of several co-morbidities, such as atherosclerosis, which hinders the determination of which CVD risk factors are exclusively associated with the kidney outcome.[63] Still, nearly 40% of hemodialysis patients presented evidence of diastolic dysfunction[64] with a high prevalence of left ventricular hypertrophy in ESRD.[65] ESRD patients with impaired diastolic function were shown to have prolonged isovolumic pressure decline with normal ejection fraction.[66] Similarly, CNTL mice had prolonged diastolic time resulting in elevated MPI, independently of a LVEF impairment. Blunted potassium redistribution is typically triggered by insulin deficiency, impaired adrenergic signaling and hyperglycemia.[67] Increases in extracellular potassium leads to a delay in the conduction of the atrioventricular node and Purkinje fibers by shortening the transmembrane action potential duration and prolonging diastolic depolarization of the Purkinje fibers.[68] Such an imbalance can contribute to cardiac arrythmia, and a predisposition to both cardiac hyperexcitability and depression.[67] Likewise, CNTL mice had a decline in the heart rate and signs of arrythmia. Thus, apart from the potential hemodynamic challenges caused by kidney dysfunction, CNTL mice might have also undergone an electrolytic challenge impairing cardiac function. Both long-term FO-feeding and PDX treatment prevented cardiac failure potentially by increasing cardiac output, while preserving heart rate and MPI. One limitation of this study

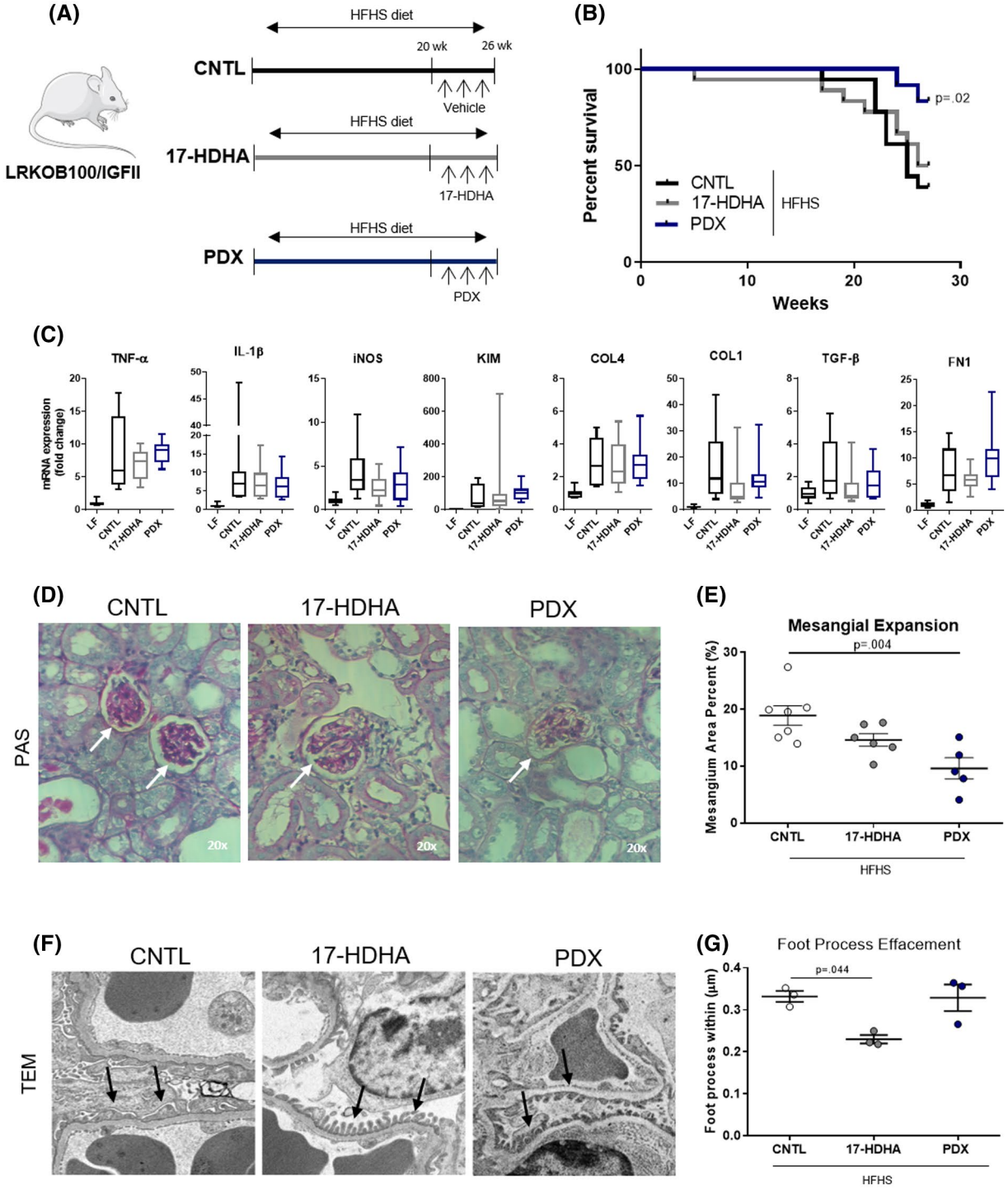

FIGURE 5 PDX treatment preserves survival rate and decreases mesangial expansion. 17-HDHA reduces podocyte foot process effacement. A, Therapeutic strategy overview. 6 weeks of gavage with PDX preserved (B) percent survival as compared to CNTL (CNTL n = 18/17-HDHA n = 18/PDX n = 12). Despite no modulations on (C) gene expression of inflammatory and pro-fibrotic factors in kidney cortex (CNTL n = 7/17-HDHA n = 8/PDX n = 8), PDX treatment was able to down-regulate mesangial expansion as seen on (D) typical images and (E) quantification (CNTL n = 7/17-HDHA n = 6/PDX n = 5). 17-HDHA further decreased podocyte foot process effacement (F, G) (CNTL n = 3/17-HDHA n = 3/PDX n = 3). Survival rate is expressed as percentage of cases, gene expression of inflammatory and fibrotic markers are presented as box plot diagram, while mesangial expansion and foot process effacement as mean ± SEM. Differences on survival rate were measured by Longrak (Mante-Cox) test *versus* CNTL. While differences on gene expression, mesangial expansion and podocyte foot process effacement were calculated using one-way ANOVA with a Dunnett's multiple comparisons post-hoc test *versus* CNTL

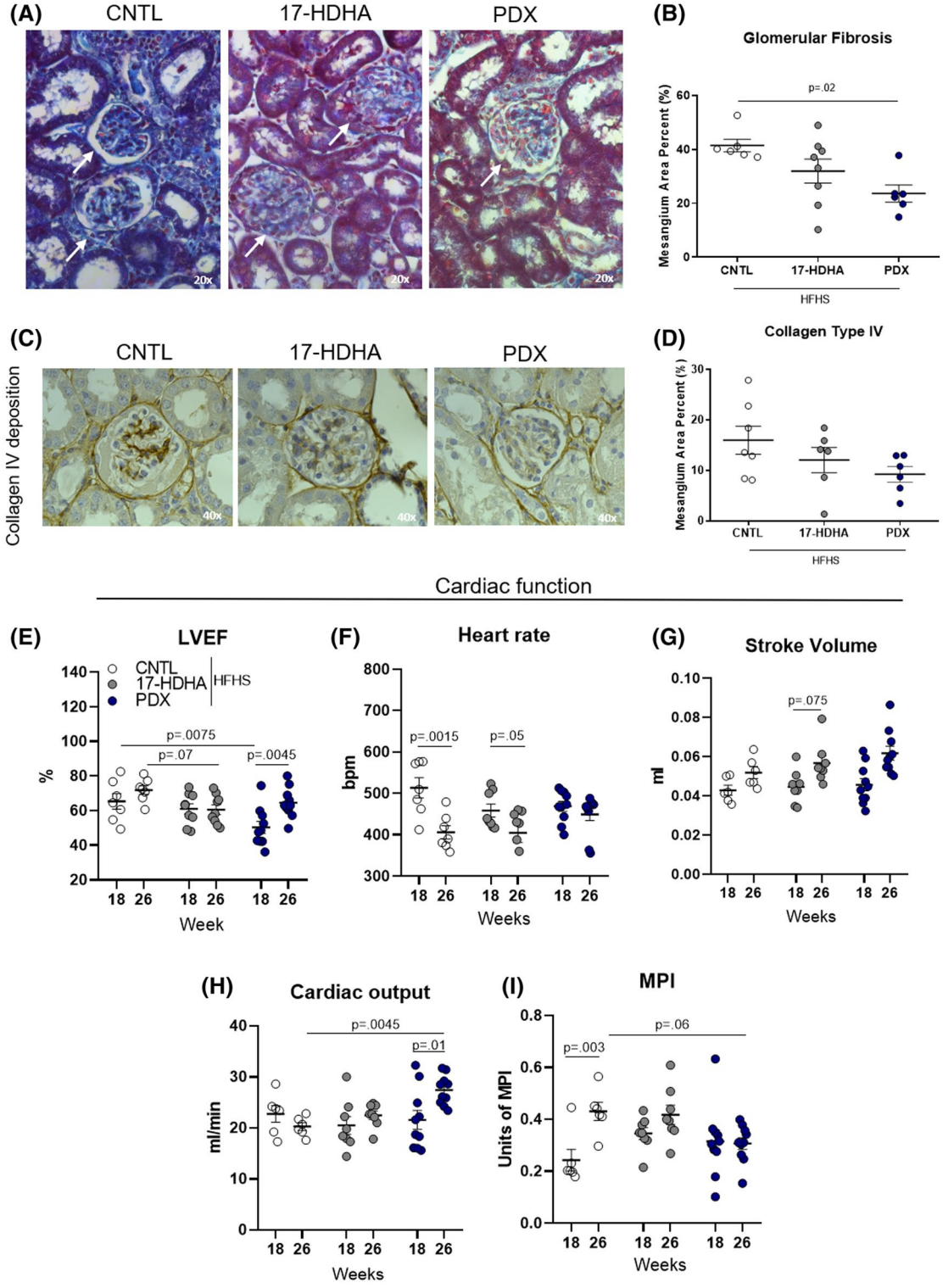

FIGURE 6 PDX treatment protects against fibrosis and prevents cardiac failure. PDX treatment was able to reduce glomerular fibrosis (A, B) as compared to CNTL (CNTL n = 6/17-HDHA n = 8/PDX n = 6). C-D, Collagen type IV was also reduced by PDX treatment (CNTL n = 7/17-HDHA n = 6/PDX n = 6), but it did not reach statistical significance. PDX treatment prevented cardiac failure by increasing (E) left ventricular ejection fraction (LVEF), as well as preserving (F) heart rate and (I) myocardial performance index (MPI). PDX was also shown to increased (H) cardiac output over time and exhibited the same trend for (G) stroke volume. (CNTL n = 6/17-HDHA n = 8/PDX n = 10). Data are expressed as mean ± SEM. Differences on glomerular fibrosis and collagen type IV deposition within the glomerulus were calculated using one-way ANOVA with a Dunnett's multiple comparisons post-hoc test *versus* CNTL. Echocardiography data was analyzed by two-way repeated measures ANOVA with a Bonferroni post hoc test *versus* CNTL

is that we have not measured blood pressure, which is also a key determinant of glomerular filtration. Indeed, consumption of omega-3 fatty acids has been reported to decrease blood pressure in older and hypertensive subjects.[69] In mice, Hoshi et al found that omega-3 fatty acids promote vasodilation via activating large-conductance Ca^{2+}- and voltage-activated K^+ channels in vascular smooth muscle cells.[70] Future studies will be needed to determine whether the beneficial effect of FO on both glomerular and cardiac profile might also involve hemodynamic changes.

We believe that the efficacy of FO and PDX treatment against ESRD progression is related to both cardiometabolic and anti-inflammatory effects. Omega-3 fatty acids are involved in the resolution phase of inflammation,[71] and we confirmed that FO exerted anti-inflammatory effects. Although we did not observe an anti-inflammatory effect of 17-HDHA and PDX in this study, a deeper evaluation of the immune cell profile may be needed to detect their anti-inflammatory potential. Yet, we were able to detect a clear effect of these two resolution mediators on promoting renal protection as revealed by the glomerular status (eg, reduced mesangial expansion and glomerular fibrosis). In this regard, it is interesting to note that both FO and PDX treatment compare favorably to commonly used therapies against DN, such as sodium–glucose co-transporter 2 (SGLT2) and renin–angiotensin–aldosterone system (RAAS) inhibitors.[72,73] Glomerular injury might be targeted by multiple physiological perturbations, including electrolytic imbalance, persistent hyperglycemia and hypertension.[74] Further studies are clearly warranted to further decipher the mechanisms underlying the beneficial effects of FO and PDX on DN.

In conclusion, our data demonstrate that dietary FO-replacement prevented features of ESRD and cardiac failure development in a mouse model of severe T2D, supporting the maintenance of omega-3 fatty acids in nutritional recommendations for prevention of diabetic kidney disease. We also provide further evidence of the therapeutic benefits of DHA-derived resolution mediators and particularly PDX against glomerular fibrosis and cardiac dysfunction, independently from its previously documented anti-diabetic properties.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

AUTHOR CONTRIBUTIONS

LRP, PLM, PS-P, and AM designed the study. LRP performed most of the experiments, with the valuable assistance of FZ and PLM. JT and OB carried out the lipidomic analysis. PM oriented echocardiography data discussion. PLM and BAHJ provided guidance and helped drafting manuscript preparation which was then revised by AM and PG. All authors were implicated in the discussion of the data and revised the final manuscript.

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SUPPORTING INFORMATION

Additional Supporting Information may be found online in the Supporting Information section.

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