Cyclooxygenase-2 disruption predisposes to heart failure with preserved ejection fraction

Summary: COX-2 genetic deletion or pharmacological inhibition results in heart failure with preserved ejection fraction in zebrafish, mice and humans.

Emanuela Ricciotti [1, 2 *], Philip G. Haines [3†], Manu Beerens [4, 5], Uri Kartoun [4, 5, 6, ‡], Cecilia Castro [7], Soon Yew Tang [2], Soumita Ghosh [2], Ujjalkumar S. Das,[2] Nicholas F. Lahens [2], Tao Wang [3, §], Jules L. Griffin [7], Stanley Y. Shaw [4, 5, 6, ‖], Calum A. MacRae [4, 5, 8] and Garret A. FitzGerald [1, 2, 9 *]

[1] Department of Systems Pharmacology and Translational Therapeutics, University of Pennsylvania Perelman School of Medicine; Philadelphia, PA 19104, USA.
[2] Institute for Translational Medicine and Therapeutics, University of Pennsylvania Perelman School of Medicine; Philadelphia, PA 19104, USA.
[3] Division of Cardiovascular Medicine, Hospital of the University of Pennsylvania; Philadelphia, PA.
[†] Present address: Rhode Island Hospital, Warren Alpert Medical School of Brown University; Providence, RI.
[4] Harvard Medical School; Boston, MA, USA.
[5] Cardiovascular Division, Brigham and Women's Hospital, Harvard Medical School; Boston, USA.
[6] Center for Systems Biology; Center for Assessment Technology & Continuous Health (CATCH), Massachusetts General Hospital; Boston, MA, USA.
[‡] Present address: Center for Computational Health, IBM Research; Cambridge, MA, USA.
[7] Rowett Institute, University of Aberdeen; Aberdeen, UK.
[§] Present address: Stanford University; Stanford, CA, USA.
[‖] Present address: One Brave Idea, Division of Cardiovascular Medicine, Brigham and Women's Hospital; Boston, MA, USA.
[8] Cardiovascular Medicine, Genetics and Network Medicine Divisions, Department of Medicine, Brigham and Women's Hospital, Harvard Medical School; Boston, USA.
[9] Department of Medicine, University of Pennsylvania Perelman School of Medicine; Philadelphia, PA 19104, USA.
[*] To whom correspondence should be addressed:
emanuela@pennmedicine.upenn.edu (E.R.); garret@upenn.edu (G.A.F.)

Abstract

Heart failure (HF) is the adverse cardiovascular event most strongly associated with cyclooxygenase (COX)-2 selective and non-selective nonsteroidal anti-inflammatory drug (NSAID) use. However, it is unknown whether NSAID exposure results in HF with reduced (HFrEF) or preserved ejection fraction (HFpEF).

In adult mice, postnatal genetic deletion or pharmacological inhibition of COX-2 did not impair cardiac function. In contrast, aged female inducible (i)Cox-2 KO mice, compared to age- and sex- matched control mice, presented diastolic dysfunction, cardiac hypertrophy, pulmonary congestion, elevated plasma N-terminal pro B-type natriuretic peptide (BNP), while ejection fraction (EF) was preserved (≥ 50%). No such phenotype was observed in aged male iCox-2 KO mice. In aged female iCox-2 KO mice, we detected a rediversion of urinary prostaglandin metabolites toward leukotriene biosynthesis and an altered expression of calcium handling proteins in the myocardium. The phospholamban to SERCA2a ratio was augmented, inferring an inhibitory impact on SERCA2a activity.

In larval zebrafish, COX-2 inhibition by celecoxib caused a modest but significant reduction in heart rate and diastolic function, while EF was preserved. Furthermore, celecoxib increased *Bnp* expression and the amplitude of ventricular Ca^{2+} transients.

In the Harvard-Partners electronic medical record (EMR), diabetic patients exposed to NSAIDs selective for COX-2 inhibition were more likely to associate with an increased risk for HFpEF than HFrEF.

Collectively, these results indicate that COX-2 deletion or inhibition does not impair systolic cardiac function, but results in a HFpEF phenotype in mice, zebrafish and humans. An imbalance

of calcium handling mediates the impairment of myocardial relaxation consequent to COX-2 disruption.

Introduction

Nonsteroidal anti-inflammatory drugs (NSAIDs) exert their pharmacological effects by reducing the biosynthesis of prostaglandins (PGs) and thromboxane (Tx) through the inhibition of cyclooxygenase (COX)-1 and COX-2. They are effective in reducing pain, inflammation, and fever. However, their use is limited by gastrointestinal (GI) and cardiovascular (CV) complications (*1, 2*).

Traditional NSAIDs (tNSAIDs), such as ibuprofen and naproxen, inhibit both COX isozymes. NSAIDs selective for COX-2 inhibition (coxibs), such as rofecoxib and celecoxib, were initially developed to reduce the incidence of serious upper GI complications but resulted in an increased incidence of adverse CV events (*3, 4*).

The Coxib and traditional NSAID Trialists' (CNT) Collaboration performed a meta-analysis of over 600 randomized clinical trials and reported that the use of coxibs and tNSAIDs, except naproxen, resulted in a roughly 30% increased risk of major vascular events (*5*). Moreover, heart failure (HF) was the adverse CV event most strongly associated with both coxibs and tNSAIDs, including naproxen, increasing the risk for hospitalization due to HF by approximately two-fold (*5*). These data are consistent with epidemiological observations (*6-11*) and randomized controlled trials of coxibs (*12*) and tNSAIDs also selective for COX-2 inhibition (*13*). In addition, the use of NSAIDs in patients with chronic HF increased the risk of CV adverse events, including an increased risk of recurrent hospitalization due to myocardial infarction (MI) or HF and a dose-dependent increase in the risk of death (*8*).

The increased risk for atherothrombotic events associated with the use of NSAIDs is mechanistically consistent with vascular COX-2 inhibition and reduced formation of cardioprotective prostanoids (*14*). However, the mechanism underlying the association between NSAID exposure and HF risk is still unexplored. The differential impact of naproxen, which causes a prolonged and persistent inhibition of platelet COX-1 like aspirin, on atherothrombotic events and HF risk indicates that NSAIDs may cause HF through a COX-2 dependent hazard unrestrained by variable COX-1 dependent platelet inhibition (*5*).

HF is a heterogeneous clinical syndrome which may be classified by left ventricular ejection fraction (EF) (*15, 16*). The predominant types are HF with reduced EF (HFrEF; EF≤ 40%) and preserved EF (HFpEF; EF≥ 50%). HFrEF, also known as systolic HF, occurs when the left ventricle (LV) loses the capacity to contract normally. HFpEF, also known as diastolic HF, occurs when the LV loses the capacity to relax normally. HFrEF and HFpEF are equally common but they differ in etiology, pathophysiology, clinical manifestations and response to therapy (*17*).

The prevalence of HF in the general population increases with age as does hospital readmission due to HF (*18, 19*). Elderly patients exposed to NSAIDs exhibit an increased risk of hospitalization (*9, 20*) and death due HF, and recurrent congestive HF (*21, 22*).

Sex-specific differences in cardiac structure, function and reserve capacity are consistent with the observation that HFrEF is more prevalent in men, while HFpEF is more prevalent in women (*23*).

It remains unknown which type of HF is associated with NSAID exposure and whether this relationship is modified by age or sex. Here, we report that NSAIDs selective for COX-2 inhibition increase the likelihood of diabetic patients presenting with HFpEF rather than HFrEF.

COX-2 deletion or inhibition results in diastolic dysfunction in aged female mice and in zebrafish. The HFpEF phenotype in mice results from unbalanced calcium handling, which contributes to the lack of myocardium relaxation.

Results

Cox-2 deletion or inhibition does not affect cardiac function in adult mice

Genetic and pharmacological manipulations in mice have recapitulated the increased predisposition to thrombosis, hypertension, atherosclerosis consequent to the suppression of COX-2 dependent prostanoids by NSAIDs (*14, 25*). Therefore, to determine the role of COX-2 on cardiac function, we performed a comprehensive functional and structural phenotyping of the heart of mice in which the COX-2 was deleted or inhibited.

We examined post-natal tamoxifen inducible Cox-2 KO mice (iCox-2 KO; *25*) and C57BL/6 mice fed with a diet containing celecoxib (100 mg/Kg) or rofecoxib (50 mg/Kg) (*26*). iCox-2 KO mice were previously generated to bypass the multiple abnormalities (including kidney defects, cardiac fibrosis and premature death) evident in conventional Cox-2 KOs (*27*).

Since HF risk is already evident during the first months of NSAID therapy (*28*), we phenotyped the mice approximately 2 months after COX-2 deletion or inhibition.

In adult female mice, the plasma concentration of celecoxib was 0.35± 0.02 μM (n=8) and 0.39± 0.04 μM (n=10) of rofecoxib. The corresponding concentrations in adult male mice were 0.50± 0.10 μM (n=8) of celecoxib and 0.48± 0.05 μM (n=10) of rofecoxib. The drug concentrations did not differ between the sexes and are in the therapeutic range (*26*).

There was no appreciable difference in body weight (Fig. S1A) and in parameters of systolic (EF) or diastolic (E/e' ratio, a surrogate marker of LV diastolic filling pressures) function or

structural remodeling between adult female and male iCox-2 KO mice compared to their age and sex-matched WT controls (Fig. S1 B-C; Table S1 and S2). Additionally, Cox-2 deletion did not affect baseline systolic blood pressure (SBP) in adult female and male mice as measured by telemetry (Fig. S1D), as was previously reported using a tail-cuff system (*25*).

Similarly, we did not observe an effect of celecoxib or rofecoxib on body weight (Fig. S1E) or on parameters of cardiac function and structural remodeling in adult female and male mice compared to age and sex matched mice on a control diet (Fig. S1, F-G, Table S3 and S4). SBP was not changed by the two coxibs in adult female and male mice (Fig. S1H), as previously reported (*26*).

Collectively, these results indicate that in adult mice, in the absence of other stressors, suppression of COX-2 alone is not sufficient to impair the cardiac function or increase blood pressure.

Cox-2 deletion impairs diastolic function in aged female mice

Since aging is one of major risk factors for HF (*18, 19*), we characterized cardiac function in approximately 2-year-old female and male iCox-2 KO mice and their age and sex-matched WT controls.

In aged mice, Cox-2 deletion did not affect body weight (Fig. 1A). Aged iCox-2 KO female mice exhibited preserved EF and a prolonged E/e` ratio, indicating diastolic dysfunction (Fig. 1, B-C). Other cardiac parameters did not differ between aged WT and iCox-2 KO female mice (Table S5). Increased LV end diastolic pressure (EDP), measured by invasive hemodynamics, both at rest and after dobutamine, corroborated the phenotype of diastolic dysfunction observed in aged iCox-2 KO female mice (Fig. 1, D-G, Table S6). Both echocardiographic and invasive

hemodynamic data failed to reveal cardiac dysfunction in aged iCox-2 KO male mice (Fig. 1, B-G, Table S7 and S8).

Pulse wave velocity (PWV), a measure of vascular stiffness, was unaltered by Cox-2 deletion in both aged female and male mice (Fig. 1H, Table S6 and S8). Consistently, SBP was unaltered by Cox-2 deletion in aged female and male mice (Fig. 1I).

As compared to their sex and age-matched WT controls, aged iCox-2 KO female mice, but not iCox-2 KO male mice, showed increased cardiac hypertrophy (as measured by heart/body weight ratio; Fig. 1J) and pulmonary congestion (as measured by lung/body weight ratio; Fig. 1K). Cox-2 deletion did not affect the presence of myocardial fibrosis (Fig. 1, L-M) in male or female mice. N-terminal (NT)-pro hormone BNP (NT-pro-BNP) levels were statistically significant elevated in female but not in aged iCox-2 KO male mice (Fig. 1N).

In summary, aged iCox-2 female KO mice present features of the human HFpEF syndrome, including preserved EF (≥50%), diastolic dysfunction, cardiac hypertrophy, pulmonary congestion, and elevated NT-pro-BNP.

Cox-2 deletion rediverts arachidonic acid metabolism in aged female mice

We confirmed that aged iCox-2 female and male KO mice achieved similar Cox-2 deletion in different organs. In aged iCox-2 female KOs, Cox-2 mRNA gene expression was reduced approximately 2-fold in the heart, 3-fold in the aorta, 4-fold in the medulla and 2-fold in the cortex, compared to age-matched female WT mice (Fig. S2 A-D). In aged iCox-2 male KOs, Cox-2 mRNA gene expression was reduced approximately 2-fold in the heart, 1.5-fold in the aorta, 4-fold in the medulla and 3-fold in the cortex, compared to their age-matched male WT

mice (Fig. S2 A-D). In aged iCox-2 female and male KOs, Cox-1 mRNA gene expression was not changed by Cox-2 deletion (Fig. S2 E-H).

Consistently, aged iCox-2 KOs exhibited over 60% reduction of the urinary PGE_2 metabolite (PGEM) and PGD_2 metabolite (PGDM), in both female and male mice (Fig. 2A and B). The urinary metabolite of TxB_2 (TXBM) was reduced by 50% only in aged iCox-2 KO male mice (Fig. 2 C). In both female and male mice, Cox-2 deletion did not detectably reduce the already low PGI_2 metabolite (PGIM) levels detected in the urine of these mice in an unchallenged condition (Fig. 2D). In aged female Cox-2 KO mice, the COX-2 substrate rediverted toward 5-lipoxygenase (LOX) dependent leukotriene (LT)E_4 formation (Fig. 2E). Other urinary metabolites of arachidonic acid (AA), linoleic acid, eicosapentaenoic acid and docosahexaenoic acid were not changed by Cox-2 deletion in male and female aged mice (Fig. 2F).

Together, these results indicate that, while aged female and male iCox-2 KOs present a similar suppression of the COX derived metabolites under basal conditions, only aged female iCox-2 KO mice reveal a rediversion of COX-2 substrate metabolism to LTE_4.

Cox-2 deletion does not impair kidney function in aged mice

In the kidney, Cox-2 is expressed both in the cortex and in the medulla (Fig. S2) and it regulates renal blood flow, glomerular filtration rate, and water and sodium extraction (*29*). Since kidney dysfunction is present in about 50% of the HFpEF patients and is associated with higher mortality (*30*), we examined kidney function and structure in aged iCox-2 KO female and male mice and their age-matched controls. In aged female and male mice, Cox-2 deletion did not reduce creatinine, albumin, and nitrate/nitrite levels, and fractional extraction of sodium (Fig. S3 A-F). Furthermore, the expression of genes relevant to the renin aldosterone angiotensin system

(RAAS) in the cortex (Fig. S3 G-J) and in the medulla (Fig. S3 K-N) were not affected by Cox-2 deletion in aged mice. Additionally, overall kidney histology was not altered by Cox-2 suppression in aged mice (Fig. S3 O-R).

In sum, these results indicate that, in absence of additional stressors, Cox-2 deletion is not sufficient alone to impair kidney function in aged mice.

Cox-2 deletion alters the expression of cardiac mitochondrial genes in aged female mice

To uncover the mechanisms by which Cox-2 deletion contributes to diastolic dysfunction in aged female hearts, we performed an unbiased transcriptomic profiling of cardiac samples harvested from aged iCox-2 female KO and aged-matched female WT controls. RNA sequencing revealed a total of 494 transcripts differentially expressed between WT and iCox-2 KO female mice at a $q < 0.355$. Seventy-six per cent of them were more highly expressed in the hearts of aged iCox-2 KO female mice, while twenty-four per cent of them were more highly expressed in the hearts of aged WT female mice (Fig. 3A). Ingenuity Pathway Analysis of the differentially expressed genes between WT and iCox-2 KO revealed an enrichment in the following pathways: oxidative phosphorylation (OXPHOS), mitochondrial dysfunction, Granzyme A signaling, Sirtuin Signaling Pathway and Glutathione-mediated detoxification (Fig. 3B). Mitochondrial dysfunction of cardiac muscle has also been reported in HFpEF (*31*). Among the mitochondrial dysfunction pathway genes, transcripts related to energy metabolism (ATP synthase, cytochrome c oxidase, NADH:ubiquinone oxidoreductase, short chain dehydrogenase/reductase) and antioxidant activity (glutathione transferases and superoxide dismutase) were down-regulated in the hearts of aged iCox-2 KO female mice. In contrast, three transcripts encoding NADH dehydrogenase subunits were up-regulated by Cox-2 deletion (Fig. 3C).

All together, these data are consistent with the hypothesis that mitochondrial dysfunction in cardiac muscle contributes to the HFpEF phenotype observed in aged iCOX-2 KO female mice.

Cox-2 deletion does not impair energy metabolism and oxidative stress in aged mice

In the heart, mitochondrial dysfunction can impair energy supply, enhance oxidative stress and disrupt calcium handling. Since disturbances in cardiac metabolism underlie most CV diseases, including HFpEF (*32*), we performed nontargeted metabolomics of the plasma, heart, aorta, kidney cortex and kidney medulla samples collected from aged iCox-2 KO female and male mice and their sex- and aged- matched WT mice. We measured aqueous metabolites covering different functions (amino acids, nucleotides, intermediates of the TCA Cycle, intermediates of the urea cycle and markers for the oxidative stress), intact lipids (triglycerides and phospholipids in particular) and acyl-carnitines. The heatmaps failed to detect genotype-dependent alterations in plasma and tissue metabolomic profiles in either female (Fig. S4) and male aged mice (Fig. S5), as previously reported in adult mice (*26*). In sum, these results indicate that Cox-2 deletion does not affect energy metabolism and oxidative stress levels in aged mice.

Cox-2 deletion imbalances calcium handling in the heart of aged female mice

Since we did not detect an impact of mitochondrial dysfunction on cardiac energy metabolism and oxidative stress by metabolomics, we investigated the effect of altered mitochondrial homeostasis on calcium handling in the heart. Interestingly, the Rnaseq analysis revelated that the transcript encoding for phospholamban (Pln), a protein that inhibits cardiac muscle sarco(endo)plasmic reticulum Ca^{2+-}ATPase (SERCA2), is more highly expressed in the hearts of aged iCox-2 KO female mice than in those of age-matched WT female mice (Fig. 4A). We

confirmed the differential expression of Pln between iCox-2 KOs and WTs in the heart of aged female mice but not in male mice by RT-PCR (Fig. 4B). A similar effect was not seen in the hearts of adult male and female mice (Fig. 4C). We then investigated the protein expression of key mediators of cardiomyocyte relaxation: SERCA2 and PLN (both in the unphosphorylated and at phosphorylated state). Only unphosphorylated PLN inhibits SERCA-2 activity and therefore impairs cardiac relaxation by preventing the cytoplasmatic calcium import into the sarcoplasmic reticulum. We found that the ratio of phosphorylated to total PLN was decreased in the hearts of aged iCox-2 KOs female mice compared aged-matched WT female controls (Fig. 4 D-F). Cox-2 deletion did not change SERCA-2 expression (Fig. 4G) but increased the PLN/SERCA ratio (Fig. 4H) in aged female hearts. Similar changes were not seen in the hearts of aged male mice (Fig. 4 D-H).

In summary, these data reveal that Cox-2 deletion increases the level of unphosphorylated PLN that can inhibit SERCA-2 (increased PLN/SERCA-2 ratio) and thereby may impair cardiac relaxation in aged female mouse hearts.

COX-2 inhibition impairs diastolic function and cardiac calcium handling in zebrafish

To determine whether our findings were conserved across species, we investigated the effect of celecoxib, a NSAID selective for COX-2 inhibition, on cardiac performance in zebrafish larvae. Both PGE_2 and $PGF_{2\alpha}$, the only prostaglandins detected in zebrafish embryo homogenates, were suppressed by celecoxib (Fig. S6), together with total cardiac output (Fig. 5A). This was not a consequence of impaired stroke volume (Fig. 5B), but rather the result of modest, but significant reduction in heart rate (Fig. 5C). More detailed analysis revealed that celecoxib-treated embryos displayed increased relaxation time (Fig. 5D), while time to contraction was marginally

increased compared to vehicle treated embryos (Fig. 5E), indicating diastolic, but not systolic dysfunction. Accordingly, fractional area change was unaltered in either celecoxib or vehicle treated embryos (Fig. 5F), however celecoxib elevated the cardiac stress marker, brain natriuretic peptide (*nppb)* (Fig. 5G). In general, there were no differences in intracellular calcium level duration during systole and diastole (Fig. 5 I-N), but celecoxib increased ventricular (Fig. 5O) and atrial (Fig. 5P) Ca^{2+} transient amplitude compared to vehicle-treated embryos.

Overall, these data indicate that COX-2 inhibition in zebrafish does not affect systolic function but impairs diastolic function and imbalances Ca^{2+} handling as seen in the aged iCox-2 female KO mice.

Exposure to NSAIDs selective for COX-2 inhibition associates with an increased risk of HFpEF in diabetic patients

To determine the clinical relevance of our findings in mice and zebrafish, we sought to determine the type of HF associated with NSAID exposure by interrogating a sub-set of the Harvard-Partners electronic medical record (EMR; 314,292 diabetic individuals).

To identify the type of HF [preserved EF (EF≥ 50%) or reduced EF (EF< 50%)], we designed an algorithm capable of extracting EF values from clinical narrative notes. In the subjects included in this study, the HF diagnosis was made after they received their first prescription for an NSAID. A manual chart review performed by a physician indicated that our algorithm correctly classified the HF type in 97.8% of 185 randomly selected notes of HF patients.

Using a multinomial multivariable regression approach, our model revealed that, after adjusting for HF confounders, an increased HFpEF odds ratio (OR) was more prevalent in male and female subjects exposed to NSAIDs selective for COX-2 inhibition versus those exposed to non-selective NSAIDs at 12- and 24- months of follow-up (Fig. 6A). A similar trend was observed

when we restricted our analysis to subjects who did not have a prescription for diuretics, commonly recommended to HF patients, before the HF diagnosis, at both follow-up periods (Fig. 6B).

In summary, our model provides evidence for an association between exposure to COX-2 selective NSAIDs and increased risk for HFpEF in humans.

Discussion

The link between NSAIDs and HF, revealed by randomized clinical trials and population studies, is remarkably under-investigated, even though it represents the cardiovascular risk (rate ratio 2.28 according to the CNT meta-analysis; *5*) most associated with the use of these drugs. Since NSAIDs are amongst the world`s most widely consumed drugs, further elucidation of this association may have considerable clinical impact.

Previously, we have reported that mice lacking Cox-2 in cardiomyocytes (CM-Cox-2 KO) presented myocardial perivascular fibrosis, abnormal contractility, exercise intolerance, arrhythmia, and preserved systolic function (*34*). Similarly, Cox-2 deficient rats, which share developmental defects with conventional Cox-2 KO mice (*27*), present with myocardial interstitial and perivascular fibrosis, dysregulation of cardiac energy metabolism and preserved systolic function (*35*). Moreover, mice treated with diclofenac, a tNSAID selective for COX-2 inhibition (*13*), show impairment of diastolic, but not systolic function, consequent, in this case, to cardiac mitochondrial dysfunction (*36*). These pre-clinical studies consistently reported normal systolic function following COX-2 deletion or inhibition, but possible diastolic dysfunction. However, in these studies the presence of extracardiac abnormalities and the possible contribution of comorbidities were not addressed.

In this study, using a multispecies approach, we confirmed that inhibition or deletion of COX-2 alone does not impair systolic function, but it may result in diastolic dysfunction in mice and zebrafish. Moreover, the interrogation of the Harvard-Partners clinical EMR database using a multinomial multivariable regression approach, revealed an increased odds ratio for HFpEF, but not HFrEF, when subjects were exposed to COX-2 selective *vs* nonselective NSAIDs. This was evident both when we included in our analysis subjects exposed to NSAIDs after HF was diagnosed and when we restricted to our analysis to subjects who did not have a prescription for diuretics before the HF event and were exposed to NSAIDs after the HF diagnosis.

HFpEF is the most common form of HF in the elderly (*37-38*) and age-related molecular and cellular alterations have been implicated in the pathogenesis of HFpEF (*39*). Female sex is associated with a higher prevalence of HFpEF (*37, 40*) and worse clinical outcomes (*41*), indicating that sex-dependent mechanisms may be involved in its pathogenesis. These may include differences in cardiac structural adaptation to pressure overload, cardiac electrophysiology, autonomic tone and activation of adaptive and innate immunity.

Our adult Cox-2 deficient mice (4–5-month-old mice, corresponding to a human age of 40-50 years old), did not present any cardiac phenotype consequent to COX-2 deletion or inhibition. Aged Cox-2 deficient female mice (18-26 month-old mice corresponding to a human age of ~70 years old), but not aged male mice, presented several hallmarks of the human HFpEF syndrome, including impairment of diastolic function, reduction of cardiac reserve, cardiac hypertrophy, lung congestion and elevated NT-pro BNP plasma levels. In contrast to CM-Cox-2 KO mice (*34*), aged iCox-2 KO mice did not exhibit increased fibrosis in the myocardium. Probably, global Cox-2 deletion in adult (*25*) and in aged mice (Fig. 1L) prevented COX-2 dependent

formation of the pro-fibrotic $PGF_{2\alpha}$ (*42*) in cardiac fibroblasts unlike what we saw in CM-Cox-2 KOs (*34*).

There are discordant findings on fibrosis in pre-clinical models of HFpEF, which often require a multi-hit approach to recapitulate fully the human condition (*43*). For example, obese ZSF1 rats, which are obese, diabetic and hypertensive, do not present with myocardial fibrosis (*44*). Moreover, fibrosis tends to be higher in patients with HFrEF than with HFpEF (*45*), and the degree of fibrosis present in HFpEF patients is highly variable (*46*), reflecting the heterogeneity within this syndrome (*47*).

Inducible deletion of Cox-2 reduced prostaglandin E_2 and D_2 biosynthesis, as reflected by urinary metabolites in both male and female aged mice, while thromboxane biosynthesis was reduced only in males, compared to age- and sex- matched control mice (Fig. 2). However, only aged female iCox-2 KO mice exhibited a rediversion of the AA substrate of COX to leukotriene biosynthesis, as reflected by urinary LTE_4. Sex differences in leukotriene biosynthesis have been reported in different species and this observation is consistent with an androgen-dependent interference with the 5-LOX/5-LOX activating protein (FLAP) complex assembly, necessary for the biosynthesis of LTs (*48, 49)*. Since the reduction of prostanoids consequent to Cox-2 deletion was observed both in aged male and female mice, while the substrate rediversion to LTE_4 was detected only in aged female iCox-2 KO mice, it is likely that the reduction of prostanoids biosynthesis with the concomitant increase in LTE_4 that drives the HFpEF phenotype.

Mechanistically, the impact of COX-2 blockade on diastolic function appears to be mediated by myocardial mitochondrial dysfunction and impairment of calcium handling. In the heart, we found 494 transcripts differentially expressed between WT and iCox-2 KO female mice. RnaSeq analysis revealed that pathways related to mitochondrial dysfunction were dysregulated in the

heart of aged iCox-2 KO female mice compared to age-matched WT male controls. This impairment of mitochondrial function did not impact energy metabolism and oxidative stress at rest, but it affected Ca^{2+} intracellular dynamics. PLN was upregulated in the heart of aged female iCox-2 KO mice both at gene and protein levels of expression. GWA studies revealed that mutations in PLN are associated with HF (*50-55*).

Cox-2 deletion did not change the expression of SERCA2a or the rate of phosphorylation of PLN. In aged female iCox-2 KO mice, the ratio PLN/Serca2a was increased, indicating a higher interaction between PLN and SERCA2a, lower SERCA2a affinity for Ca^{2+}, and therefore impairment of the myocardial relaxation.

Zebrafish treated with celecoxib, at a dose that impairs diastolic but not systolic function and increases *Nppb* expression, also showed Ca^{2+} mishandling with an increase in the amplitude of calcium transients. Similarly, HFpEF rats showed augmented calcium transient amplitude and increased PLN expression in their cardiomyocytes (*56*).

In this study, the diastolic dysfunction observed in aged iCox-2 KO female mice was not the result of alterations in hemodynamics or renal function consequent to Cox-2 deletion in the kidney. This, despite kidney dysfunction being a non-cardiac comorbidity associated with HFpEF (*30*) and NSAIDs having the capacity to alter water and sodium retention and predispose to hypertension (*29*). In the absence of additional stressors, Cox-2 deletion alone was insufficient to impair renal function in aged mice and iCox-2 KO mice were not hypertensive. This is consistent with our previous work in which we reported that only celecoxib-treated mice infused with angiotensin II, at a dose sufficient to elevate BP, showed impairment of renal function and increased formation of methylarginines in the kidney (*26*).

As HFpEF is a syndrome, it is likely that multiple mechanistic pathways contribute to its expression and their manipulation might afford preclinical models of the condition. The clinical and experimental evidence provided in this study indicate that, in absence of comorbidities, COX-2 deletion or inhibition does not impair systolic function, but it may alter diastolic function. COX-2 blockade causes features of the HFpEF phenotype in aged female mice and zebrafish. Mitochondrial dysfunction and impairment of calcium handling appear to contribute to the impaired myocardial relaxation consequent to COX-2 disruption.

Limitations

A limitation of the mouse study is the small sample size in some experiments due to the challenge of working with 2-year-old mice. Although we did not observe an effect of Cox-2 deletion on survival, some procedures performed in aged mice (e.g. telemetry implantation) cause a high mortality rate (around 70 %) in both genotypes. For the same reason, we could not perform exercise tolerance tests in these mice, although exercise intolerance is a feature reported in HFpEF patients. Although we reported an association between female sex and the HFpEF phenotype in mice, we cannot claim causation since we did not investigate the effect of sex hormones. The zebrafish study does not elaborate the contribution of sex and age on the diastolic dysfunction phenotype.

The observational nature of the EMR analysis prevented us from being able to control for all potential confounding variables and the HF phenotype was not confirmed with biomarker studies. Furthermore, the EMR cohort lacks a control group not exposed to NSAIDs and confirmation of drug exposure in the patients exposed to NSAIDs.

A limitation of the translational impact of the current study is that the EMR population was diabetic and therefore was at higher risk for metabolic disease and/or cardiovascular disease,

while COX-2 deletion or inhibition was performed in normoglycemic model organisms. Future studies are warranted to investigate the effect of COX-2 deletion or inhibition on cardiac function in the presence of aging, metabolic disease, obesity and/or hypertension to incorporate prevalent comorbidities that may be present in regular NSAID users.

Methods summary

The aim of the EMR study was to investigate the type of HF, with EF≥ 50 % or EF<50%, associated to COX-2 selective NSAIDs or nonselective NSAID exposure by interrogating large human observational databases using a multinomial multivariable regression approach.

The aim of the studies in model organism was to investigate the effect of COX-2 deletion and inhibition on cardiac function. In mice, we characterized the cardiac function by echocardiography, invasive hemodynamic and by measuring blood pressure by telemetry. Mechanistic studies included RNAseq, urine lipidomic profiling, plasma, heart, aorta, and kidney metabolome profiling, Wester-blots. In zebrafish, we assessed cardiac performance, measured Ca^{2+} dynamics, and evaluated *nppb* expression by luciferase assay. Inhibition of prostaglandin biosynthesis *in vivo* and plasma celecoxib levels were measured by mass spectroscopy.

Sample sizes were empirically determined based on our previous experience with the animal models used (14, 25-26, 34). All animals were randomly allocated to the treatment groups, and the experimenters were blinded to such allocation. The exact number of animals, their sex and age in each experiment is provided in the respective figures. Please refer to Supplementary Materials and Methods for further details on experimental procedures.

Statistical analysis

Data are presented as bar with error bars showing means and SEM, respectively, with single data points superimposed. Indicated sample sizes in figure legends refer to biological replicates. Comparisons between two groups were done by Mann-Whitney's t test. In the case of multiple group comparisons, analysis of variance (ANOVA) followed by Sidak`s post hoc tests were applied.

Statistical analysis of EMR data and omics data are described in detail within their respective methods sections in Supplementary Materials and Methods. All reported P values are two-sided, and an α level of 0.05 was used throughout.

Unless stated otherwise, statistical analyses were performed using R package (version 3.6.0) or GraphPad Prism 10 (GraphPad Software LLC, Massachusetts, USA).

List of Supplementary Materials

Material and Methods

Fig. S1. Cox-2 deletion or inhibition does not impair cardiac function in adult male and female mice.
Fig. S2. Cox-2 deletion determines a comparable suppression of the COX-2 pathway in aged female and male mice.
Fig. S3. Aged iCox-2 KO female and male mice do not present impairment of kidney function.
Fig. S4. Cox-2 deletion does not impair energy metabolism and oxidative stress in aged female mice.
Fig. S5. Cox-2 deletion does not impair energy metabolism and oxidative stress in aged male mice.
Fig. S6. Levels of celecoxib, PGE2 and PGF2□ in zebrafish homogenizes.
Table S1. Echo parameters in adult female iCox-2 KO and WT mice.
Table S2. Echo parameters in adult male iCox-2 KO and WT mice.
Table S3. Echo parameters in adult female mice on celecoxib or rofecoxib or control diet.
Table S4. Echo parameters in adult male mice on celecoxib or rofecoxib or control diet.
Table S5. Echo parameters in old female iCox-2 KO and WT mice.
Table S6. Invasive hemodynamic parameters in old female iCox-2 KO and WT mice.
Table S7. Echo parameters in old male iCox-2 KO and WT mice.
Table S8. Invasive hemodynamic parameters in old male iCox-2 KO and WT mice.
Table S9. NSAIDs selective for COX-2 inhibition prescriptions extracted from the EMR database.

Table S10. Nonselective NSAID prescriptions extracted from the EMR database.
Table S11. Diagnosis codes used to identify comorbidities in the EMR database.
Table S12. Diuretics prescriptions extracted from the EMR database.
References (*57-62*).

References

1. Bjarnason I, Scarpignato C, Holmgren E, Olszewski M, Rainsford KD, Lanas A. Mechanisms of Damage to the Gastrointestinal Tract From Nonsteroidal Anti-Inflammatory Drugs. Gastroenterology. 2018;154(3):500-514.
2. Grosser T, Ricciotti E, FitzGerald GA. The Cardiovascular Pharmacology of Nonsteroidal Anti-Inflammatory Drugs. Trends Pharmacol Sci. 2017;38(8):733-748.
3. Silverstein FE, Faich G, Goldstein JL, Simon LS, Pincus T, Whelton A, Makuch R, Eisen G, Agrawal NM, Stenson WF, Burr AM, Zhao WW, Kent JD, Lefkowith JB, Verburg KM, Geis GS. Gastrointestinal toxicity with celecoxib vs nonsteroidal anti-inflammatory drugs for osteoarthritis and rheumatoid arthritis: the CLASS study: A randomized controlled trial. Celecoxib Long-term Arthritis Safety Study. JAMA. 2000;284(10):1247-55.
4. Bombardier C, Laine L, Reicin A, Shapiro D, Burgos-Vargas R, Davis B, Day R, Ferraz MB, Hawkey CJ, Hochberg MC, Kvien TK, Schnitzer TJ; VIGOR Study Group Comparison of upper gastrointestinal toxicity of rofecoxib and naproxen in patients with rheumatoid arthritis. VIGOR Study Group. N Engl J Med. 2000;343(21):1520-8
5. Coxib and traditional NSAID Trialists' (CNT) Collaboration, et al. Vascular and upper gastrointestinal effects of non-steroidal anti-inflammatory drugs: meta-analyses of individual participant data from randomised trials. Lancet 2013; 382:769-79.

6. Ungprasert P, Srivali N, Kittanamongkolchai W. Non-steroidal anti-inflammatory drugs and risk of heart failure exacerbation: A systematic review and meta-analysis. Eur J Intern Med. 2015;26(9):685-90.

7. García Rodríguez LA and Hernández-Díaz S. Nonsteroidal antiinflammatory drugs as a trigger of clinical heart failure. Epidemiology 2003;14:240-246.

8. Gislason GH, Rasmussen JN, Abildstrom SZ, Schramm TK, Hansen ML, Fosbøl EL, Sørensen R, Folke F, Buch P, Gadsbøll N, Rasmussen S, Poulsen HE, Køber L, Madsen M, Torp-Pedersen C. Increased mortality and cardiovascular morbidity associated with use of nonsteroidal anti-inflammatory drugs in chronic heart failure. Archives of internal medicine. 2009; 169:141-149.

9. Arfè A, Scotti L, Varas-Lorenzo C, Nicotra F, Zambon A, Kollhorst B, Schink T, Garbe E, Herings R, Straatman H4, Schade R5, Villa M6, Lucchi S6, Valkhoff V5, Romio S5, Thiessard F7, Schuemie M5, Pariente A7, Sturkenboom M5, Corrao G8; Safety of Non-steroidal Anti-inflammatory Drugs (SOS) Project Consortium. Non-steroidal anti-inflammatory drugs and risk of heart failure in four European countries: nested case-control study. BMJ. 2016;354:i4857.

10. Scott PA, Kingsley GH, Scott DL. Non-steroidal anti-inflammatory drugs and cardiac failure: meta-analyses of observational studies and randomised controlled trials. Eur J Heart Fail. 2008;10(11):1102-7.

11. Holt A, Strange JE, Nouhravesh N, Nielsen SK, Malik ME, Schjerning AM, Køber L, Torp-Pedersen C, ison GH, McGettigan P, Schou M, Lamberts M. Heart Failure Following Anti-Inflammatory Medications in Patients With Type 2 Diabetes Mellitus. J Am Coll Cardiol. 2023;81(15):1459-1470.

12. Grosser T, Yu Y, Fitzgerald GA. Emotion recollected in tranquility: lessons learned from the COX-2 saga. Annu Rev Med 2010, 61, 17-33.

13. Grosser T, Fries S and Fitzgerald GA. Biological basis for the cardiovascular consequences of COX-2 inhibition: therapeutic challenges and opportunities. J Clin Invest 2006; 116:4-15.

14. Yu Y, Ricciotti E, Scalia R, Tang SY, Grant G, Yu Z, Landesberg G, Crichton I, Wu W, Puré E, Funk CD, FitzGerald GA. Vascular COX-2 modulates blood pressure and thrombosis in mice. Sci Transl Med. 2012;4(132):132ra54.

15. Classification of Heart Failure According to Ejection Fraction: JACC Review Topic of the Week. Lam CSP, et al. J Am Coll Cardiol. 2021; 29;77(25):3217-3225.

16. Heidenreich PA, Bozkurt B, Aguilar D, Allen LA, Byun JJ, Colvin MM, Deswal A, Drazner MH, Dunlay SM, Evers LR, Fang JC, Fedson SE, Fonarow GC, Hayek SS, Hernandez AF, Khazanie P, Kittleson MM, Lee CS, Link MS, Milano CA, Nnacheta LC, Sandhu AT, Stevenson LW, Vardeny O, Vest AR, Yancy CW. 2022 AHA/ACC/HFSA Guideline for the Management of Heart Failure: A Report of the American College of Cardiology/American Heart Association Joint Committee on Clinical Practice Guidelines. J Am Coll Cardiol. 2022;79(17):e263-e421.

17. Stoicescu L, Crişan D, Morgovan C, Avram L, Ghibu S. Heart Failure with Preserved Ejection Fraction: The Pathophysiological Mechanisms behind the Clinical Phenotypes and the Therapeutic Approach. Int J Mol Sci. 2024;25(2):794.

18. Roger VL. Epidemiology of Heart Failure: A Contemporary Perspective. Circ Res. 2021 ;128(10):1421-1434.

19. Corrao G, Ghirardi A, Ibrahim B, Merlino L, Maggioni AP. Short- and long-term mortality and hospital readmissions among patients with new hospitalization for heart failure: A population-based investigation from Italy. Int J Cardiol. 2015;181:81-7.

20. Mamdani M, Juurlink DN, Lee DS, Rochon PA, Kopp A, Naglie G, Austin PC, Laupacis A, Stukel TA. Cyclo-oxygenase-2 inhibitors versus non-selective non-steroidal anti-inflammatory drugs and congestive heart failure outcomes in elderly patients: a population-based cohort study. Lancet. 2004;363(9423):1751-6.

21. Hudson M, Richard H, Pilote L. Differences in outcomes of patients with congestive heart failure prescribed celecoxib, rofecoxib, or non-steroidal anti-inflammatory drugs: population based study. BMJ. 2005;330(7504):1370.

22. Hudson M, Rahme E, Richard H, Pilote L. Risk of congestive heart failure with nonsteroidal antiinflammatory drugs and selective Cyclooxygenase 2 inhibitors: a class effect? Arthritis Rheum. 2007;57(3):516-23.

23. Savarese G, D'Amario D. Sex Differences in Heart Failure. Adv Exp Med Biol. 2018;1065:529-544.

24. Bavry AA, Thomas F, Allison M, Johnson KC, Howard BV, Hlatky M, Manson JE, Limacher MC. Nonsteroidal anti-inflammatory drugs and cardiovascular outcomes in women: results from the women's health initiative. Circ Cardiovasc Qual Outcomes. 2014;7(4):603-10.

25. Yu Z, Crichton I, Tang SY, Hui Y, Ricciotti E, Levin MD, Lawson JA, Puré E, FitzGerald GA. Disruption of the 5-lipoxygenase pathway attenuates atherogenesis consequent to COX-2 deletion in mice. Proc Natl Acad Sci U S A. 2012;109(17):6727-32.

26. Ricciotti E, Castro C, Tang SY, Briggs WTE, West JA, Malik D, Rhoades SD, Meng H, Li X, Lahens NF, Sparks JA, Karlson EW, Weljie AM, Griffin JL, FitzGerald GA. Cyclooxygenase-2, Asymmetric Dimethylarginine, and the Cardiovascular Hazard From Nonsteroidal Anti-Inflammatory Drugs. Circulation. 2018;138(21):2367-2378

27. Dinchuk JE, Car BD, Focht RJ, Johnston JJ, Jaffee BD, Covington MB, Contel NR, Eng VM, Collins RJ, Czerniak PM, Gorry SA, Trzaskos JM. Renal abnormalities and an altered inflammatory response in mice lacking cyclooxygenase II. Nature. 1995;378(6555):406-9.

28. García Rodríguez LA, Hernández-Díaz S. Nonsteroidal antiinflammatory drugs as a trigger of clinical heart failure. Epidemiology. 2003;14(2):240-6.

29. FitzGerald GA. The choreography of cyclooxygenases in the kidney. J Clin Invest. 2002 ;110(1):33-4.

30. Streng KW, Nauta JF, Hillege HL, Anker SD, Cleland JG, Dickstein K, Filippatos G, Lang CC, Metra M, Ng LL, Ponikowski P, Samani NJ, van Veldhuisen DJ, Zwinderman AH, Zannad F, Damman K, van der Meer P, Voors AA. Non-cardiac comorbidities in heart failure with reduced, mid-range and preserved ejection fraction. Int J Cardiol. 2018;271:132-139.

31. Schwartz B, Gjini P, Gopal DM, Fetterman JL. Inefficient Batteries in Heart Failure: Metabolic Bottlenecks Disrupting the Mitochondrial Ecosystem. JACC Basic Transl Sci. 2022;7(11):1161-1179.

32. Hage C, Löfgren L, Michopoulos F, Nilsson R, Davidsson P, Kumar C, Ekström M, Eriksson MJ, Lyngå P, Persson B, Wallén H, Gan LM, Persson H, Linde C. Metabolomic Profile in HFpEF vs HFrEF Patients. J Card Fail. 2020;26(12):1050-1059.

33. Bowley G, Kugler E, Wilkinson R, Lawrie A, van Eeden F, Chico TJA, Evans PC, Noël ES, Serbanovic-Canic J. Zebrafish as a tractable model of human cardiovascular disease. Br J Pharmacol. 2022;179(5):900-917.

34. Wang D, Patel VV, Ricciotti E, Zhou R, Levin MD, Gao E, Yu Z, Ferrari VA, Lu MM, Xu J, Zhang H, Hui Y, Cheng Y, Petrenko N, Yu Y, FitzGerald GA Cardiomyocyte cyclooxygenase-2 influences cardiac rhythm and function. Proc Natl Acad Sci U S A. 2009;106:7548-52.

35. Wan Q, Kong D, Liu Q, Guo S, Wang C, Zhao Y, Ke ZJ, Yu Y. Congestive heart failure in COX2 deficient rats. Sci China Life Sci. 2021;64(7):1068-1076.

36. Thai PN, Ren L, Xu W, Overton J, Timofeyev V, Nader CE, Haddad M, Yang J, Gomes AV, Hammock BD, Chiamvimonvat N, Sirish P. Chronic Diclofenac Exposure Increases Mitochondrial Oxidative Stress, Inflammatory Mediators, and Cardiac Dysfunction. Cardiovasc Drugs Ther. 2023;37(1):25-37.

37. Dunlay SM, Roger VL, Redfield, MM. Epidemiology of Heart Failure with Preserved Ejection Fraction. Nat Rev Cardiol 2017;14:591-602.

38. Kitzman DW, O'Neill TJ, Brubaker PH. Unraveling the Relationship between Aging and Heart Failure with Preserved Ejection Fraction. JACC: Heart Fail. 2017: 5;356-358.

39. Abdellatif M, Kroemer G. Heart failure with preserved ejection fraction: An age-related condition. J Mol Cell Cardiol. 2022;167:83-84.

40. Kumar V, Redfield MM, Glasgow A, Roger VL, Weston SA, Chamberlain AM, Dunlay SM. Incident Heart Failure With Mildly Reduced Ejection Fraction: Frequency, Characteristics, and Outcomes. J Card Fail. 2023;29(2):124-134.

41. Sotomi Y, Hikoso S, Nakatani D, Mizuno H, Okada K, Dohi T, Kitamura T, Sunaga A, Kida H, Oeun B, Sato T, Komukai S, Tamaki S, Yano M, Hayashi T, Nakagawa A, Nakagawa Y, Yasumura Y, Yamada T, Sakata Y; PURSUIT□HFpEF Investigators. Sex Differences in Heart Failure With Preserved Ejection Fraction. J Am Heart Assoc. 2021;10(5):e018574.

42. Oga T, Matsuoka T, Yao C, Nonomura K, Kitaoka S, Sakata D, Kita Y, Tanizawa K, Taguchi Y, Chin K, Mishima M, Shimizu T, Narumiya S. Prostaglandin F(2alpha) receptor signaling facilitates bleomycin-induced pulmonary fibrosis independently of transforming growth factor-beta. Nat Med. 2009 Dec;15(12):1426-30.

43. Roh J, Hill JA, Singh A, Valero-Muñoz M, Sam F. Heart Failure With Preserved Ejection Fraction: Heterogeneous Syndrome, Diverse Preclinical Models. Circ Res. 2022;130(12):1906-1925

44. Hamdani N, Franssen C, Lourenço A, Falcão-Pires I, Fontoura D, Leite S, Plettig L, López B, Ottenheijm CA, Becher PM, González A, Tschöpe C, Díez J, Linke WA, Leite-Moreira AF, Paulus WJ. Myocardial titin hypophosphorylation importantly contributes to heart failure with preserved ejection fraction in a rat metabolic risk model. Circ Heart Fail. 2013;6(6):1239-49.

45. Su MY, Lin LY, Tseng YH, Chang CC, Wu CK, Lin JL, Tseng WY. CMR-verified diffuse myocardial fibrosis is associated with diastolic dysfunction in HFpEF. JACC Cardiovasc Imaging. 2014;7(10):991-7.

46. Hahn VS, Yanek LR, Vaishnav J, Ying W, Vaidya D, Lee YZJ, Riley SJ, Subramanya V, Brown EE, Hopkins CD, Ononogbu S, Perzel Mandell K, Halushka MK, Steenbergen C Jr, Rosenberg AZ, Tedford RJ, Judge DP, Shah SJ, Russell SD, Kass DA, Sharma K.

Endomyocardial Biopsy Characterization of Heart Failure With Preserved Ejection Fraction and Prevalence of Cardiac Amyloidosis. JACC Heart Fail. 2020;8(9):712-724.

47. Chirinos JA. Discerning the Age-Related Heterogeneity in Heart Failure With Preserved Ejection Fraction. J Am Coll Cardiol. 2019;74(5):613-616.

48. Pace S, Rossi A, Krauth V, Dehm F, Troisi F, Bilancia R, Weinigel C, Rummler S, Werz O, Sautebin L. Sex differences in prostaglandin biosynthesis in neutrophils during acute inflammation. Sci Rep. 2017;7(1):3759.

49. Pace S, Pergola C, Dehm F, Rossi A, Gerstmeier J, Troisi F, Pein H, Schaible AM, Weinigel C, Rummler S, Northoff H, Laufer S, Maier TJ, Rådmark O, Samuelsson B, Koeberle A, Sautebin L, Werz O. Androgen-mediated sex bias impairs efficiency of leukotriene biosynthesis inhibitors in males. J Clin Invest. 2017;127(8):3167-3176.

50. Cuello F, Knaust AE, Saleem U, Loos M, Raabe J, Mosqueira D, Laufer S, Schweizer M, van der Kraak P, Flenner F, Ulmer BM, Braren I, Yin X, Theofilatos K, Ruiz-Orera J, Patone G, Klampe B, Schulze T, Piasecki A, Pinto Y, Vink A, Hübner N, Harding S, Mayr M, Denning C, Eschenhagen T, Hansen A. Impairment of the ER/mitochondria compartment in human cardiomyocytes with PLN p.Arg14del mutation. EMBO Mol Med. 2021;13(6):e13074.

51. Schmitt JP, Kamisago M, Asahi M, Li GH, Ahmad F, Mende U, Kranias EG, MacLennan DH, Seidman JG, Seidman CE. Dilated cardiomyopathy and heart failure caused by a mutation in phospholamban. Science 2003; 299:1410–1413.

52. Haghighi K, Kolokathis F, Gramolini AO, Waggoner JR, Pater L, Lynch RA, Fan GC, Tsiapras D, Parekh RR, Dorn GW 2nd, MacLennan DH, Kremastinos DT, Kranias EG. A

mutation in the human phospholamban gene, deleting arginine 14, results in lethal, hereditary cardiomyopathy. Proc Natl Acad Sci USA 2006;103:1388–1393.

53. van der Zwaag PA, van Rijsingen IA, Asimaki A, Jongbloed JD, van Veldhuisen DJ, Wiesfeld AC, Cox MG, van Lochem LT, de Boer RA, Hofstra RM, Christiaans I, van Spaendonck-Zwarts KY, Lekanne dit Deprez RH, Judge DP, Calkins H, Suurmeijer AJ, Hauer RN, Saffitz JE, Wilde AA, van den Berg MP, van Tintelen JP. Phospholamban R14del mutation in patients diagnosed with dilated cardiomyopathy or arrhythmogenic right ventricular cardiomyopathy: Evidence supporting the concept of arrhythmogenic cardiomyopathy. Eur J Heart Fail 2012; 14:1199–1207

54. Medeiros A, Biagi DG, Sobreira TJP, de Oliveira PSL, Negrão CE, Mansur AJ, Krieger JE, Brum PC, Pereira AC. Mutations in the human phospholamban gene in patients with heart failure. Am Heart J 2011; 162:1088–1095.

55. Levin MG, Tsao NL, Singhal P, Liu C, Vy HMT, Paranjpe I, Backman JD, Bellomo TR, Bone WP, Biddinger KJ, Hui Q, Dikilitas O, Satterfield BA, Yang Y, Morley MP, Bradford Y, Burke M, Reza N, Charest B; Regeneron Genetics Center; Judy RL, Puckelwartz MJ, Hakonarson H, Khan A, Kottyan LC, Kullo I, Luo Y, McNally EM, Rasmussen-Torvik LJ, Day SM, Do R, Phillips LS, Ellinor PT, Nadkarni GN, Ritchie MD, Arany Z, Cappola TP, Margulies KB, Aragam KG, Haggerty CM, Joseph J, Sun YV, Voight BF, Damrauer SM. Genome-wide association and multi-trait analyses characterize the common genetic architecture of heart failure. Nat Commun. 2022;13(1):6914).

56. Kilfoil PJ, Lotteau S, Zhang R, Yue X, Aynaszyan S, Solymani RE, Cingolani E, Marbán E, Goldhaber JI. Distinct features of calcium handling and β-adrenergic sensitivity in

heart failure with preserved versus reduced ejection fraction. J Physiol. 2020;598(22):5091-5108.

Funding: This study was supported by a grant from the NIH (HL117798).

Author contributions: ER and PH designed and supervised the study; ER, and GAF wrote the manuscript; ER, MB, CC, SYT, SG, USD, JA, NJL, TW, performed experiments and analyzed and discussed data; UK and SYS interrogated and analyzed the clinical data in EMRs; ER, PH, JLG, SYS, CAMR, GAF discussed data and/or gave conceptual advice**. Competing interests:** G.A.F. is the McNeil Professor in Translational Medicine and Therapeutics and Senior Advisor to Calico Laboratories. All authors declare no competing non-financial interests. **Data and materials availability**: All data associated with this study are present in the paper or the Supplementary Materials. The RNA-seq data are accessible on the NCBI GEO database under the accession number GSE.

Figure Legends

Fig. 1. Cox-2 deletion predisposes to diastolic dysfunction in aged female mice. (A-H) Cardiac function characterization of 18-26-month-old iCox-2 KO female and male mice and sex- and age- matched WTs. A) Body weight. (B) Ejection fraction (EF). (C) Ratio of peak early Doppler transmitral flow velocity (E) to myocardial tissue Doppler velocity (e′) ratio. (D) End diastolic pressure (EDP). (E) EDP after first dose of dobutamine. (F) EDP after second dose of dobutamine. (G) EDP after third dose of dobutamine. (H) Pulse waive velocity (PWV). (I) Systolic blood pressure (BP) measured by telemetry. (J) Heart weight normalized to body weight. (K) Lung weight normalized to body weight. (L) Representative micrographs and (M) quantification of LV fibrotic remodeling because of collagen accumulation, as evaluated by

picrosirius red staining. At least eight distinct non over-lapping sections were quantified to analyze the percentage fibrosis in the heart for each genotype. (N) N-terminal (NT)-pro hormone BNP (NT-pro-BNP). P values were calculated by one-way ANOVA with Sidak`s post hoc test. Bars (red= females; blue= males) and error bars show means and SEM, respectively, with individual data points superimposed.

Fig. 2. Cox-2 deletion rediverts arachidonic acid metabolism in aged female mice. Urinary metabolites of arachidonic acid, linoleic acid, eicosapentaenoic acid and docosahexaenoic acid measured in WT and iCox-2 KO mice by liquid-chromatography mass-spectrometry and normalized by urinary creatinine levels. (A) Urinary PGE_2 metabolite (PGEM). (B) Urinary PGD_2 metabolite (PGDM). (C) Urinary TXB_2 metabolite (TXBM). (D) Urinary PGI_2 metabolite (PGIM). (E) Urinary LTE_4 metabolite. (F) Heatmap depicting relative levels (red = high, blue = low) of oxylipins in the urine of aged WT and iCOX-2 KO male and female mice. Pairwise *t* tests were used to determine significant differences between WT and iCox-2 KO within the same sex. Bars (red= females; blue= males) and error bars show means and SEM, respectively, with individual data points superimposed.

Fig. 3. **Aged iCox-2 KO female mice present cardiac muscle mitochondrial dysfunction.** RNA samples isolated from the heart of aged (18-26-month-old) WT and iCOX-2 KO female mice were used for RNA-Seq (n=5 per genotype). (A) Volcano plot showing up and down-regulated transcripts in the heart of aged iCOX-2 KO female mice compared to age matched female WTs. (B) Top canonical pathways enriched by gene differentially expressed in the heart of WT and iCOX-2 KO female mice as identified by Ingenuity Pathway Analysis. (C) Heatmap

depicting relative abundance of mitochondrial dysfunction transcripts differently expressed (red = high, blue = low) between aged WT and iCOX-2 KO female mice.

Fig. 4. Cox-2 deletion alters calcium handling in the heart of aged female mice. (A) Number of reads mapped to Pln in RnaSeq analysis of the heart of aged WT and iCOX-2 KO female mice. (B) Gene expression of Pln in the heart of aged WT and iCOX-2 KO female and male mice by RT-PCR. (C) Gene expression of Pln in the heart of adult WT and iCOX-2 KO female and male mice by RT-PCR. (D-H) Quantification of the protein expression of total PLN, phospho-PLN, SERCA2a in the heart of aged WT and iCOX-2 KO female and male mice measured by Western blotting and quantified using the ImageJ software. Pairwise *t* tests were used to determine significant differences between WT and iCox-2 KO within the same sex. Bars (red= females; blue= males) and error bars show means and SEM, respectively, with individual data points superimposed.

Fig. 5. COX-2 inhibition impairs diastolic function and unbalances cardiac calcium homeostasis in larval zebrafish. Effect of celecoxib (25 μM) on cardiac performance in zebrafish larvae (A-F). (A) Cardiac output. (B) Stroke volume. (C) Heart rate. (D) Relaxation time. (E) Contraction time. (F) Fractional area change. (G) Brain natriuretic peptide (nppb) expression measured by a luciferase assay. (H) Representative color maps of calcium dynamics. (I) Ventricular diastolic calcium concentration. (J) Atrial diastolic calcium concentration. (K) Ventricular systolic calcium concentration. (L) Atrial systolic calcium concentration. (M) Ventricular calcium duration. (N) Atrial calcium duration. (O) Ventricular calcium amplitude. (P) Atrial calcium amplitude. Pairwise *t* tests were used to determine significant differences

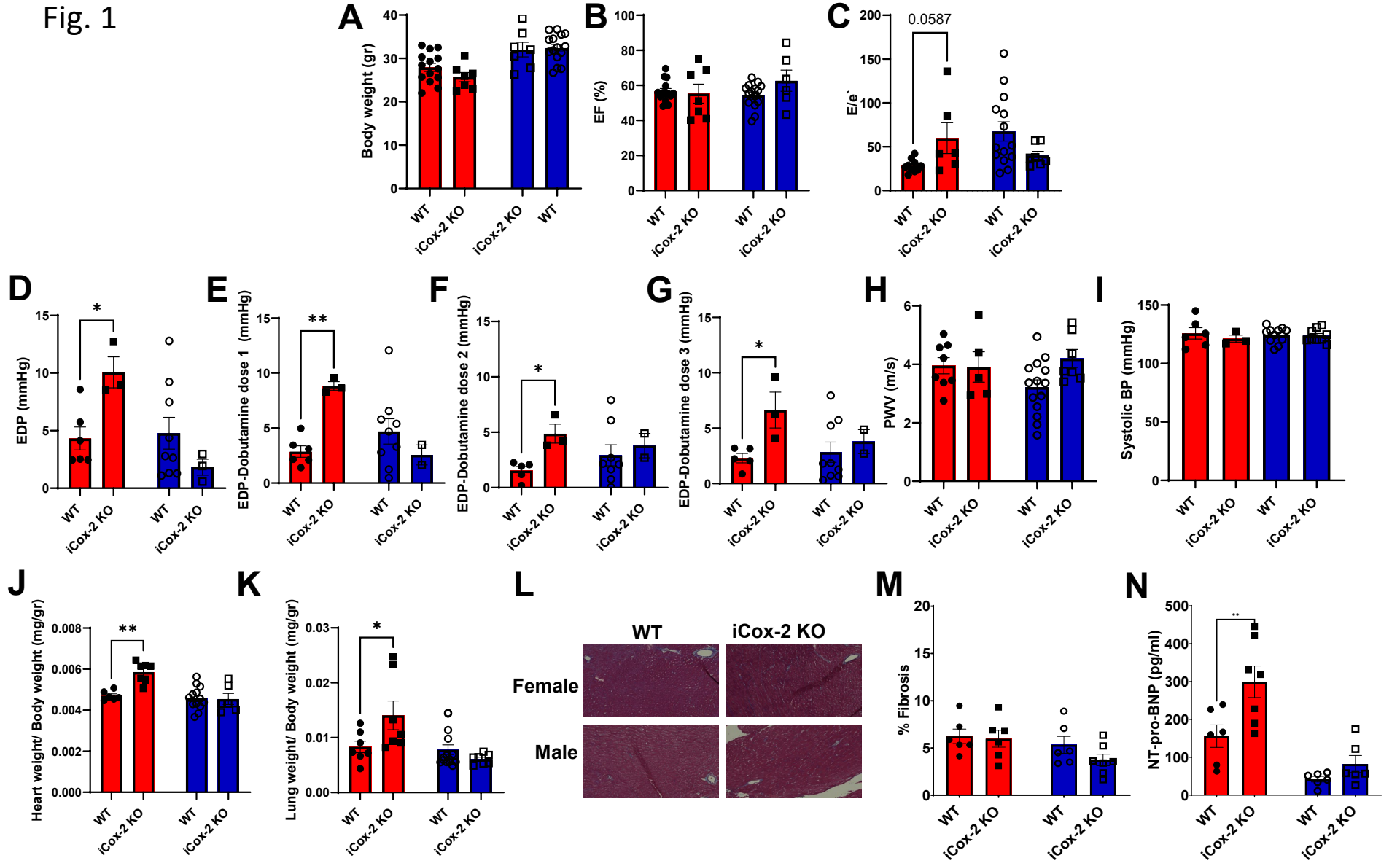
between DMSO (control) and celecoxib. Bars and error bars show means and SEM, respectively, with individual data points superimposed.

Fig. 6. Exposure to NSAIDs selective for COX-2 inhibition is associated with an increased risk of the HFpEF in humans.

(A) Association, expressed as odds ratio, between exposure to COX-2 selective NSAID versus non-selective NSAID to EF ≥ 50% (HFpEF) or EF < 50% (HFrEF) in HF patients at 12- and 24-month follow-up after the first NSAID prescription.

(B) Association, expressed as odds ratio, between exposure to COX-2 selective NSAID versus non-selective NSAID to EF ≥ 50% (HFpEF) or EF < 50% (HFrEF) in HF patients at 12- and 24-month follow-up after the first NSAID exposure. No diuretic prescriptions were allowed before HF diagnosis. Data are reported as median with range. Red dots= women; Blue dots= males.

Fig. 1


A
Body weight (gr)
B
EF (%)
C
E/e'
0.0587
D
EDP (mmHg)
E
EDP-Dobutamine dose 1 (mmHg)
F
EDP-Dobutamine dose 2 (mmHg)
G
EDP-Dobutamine dose 3 (mmHg)
H
PWV (m/s)
I
Systolic BP (mmHg)
J
Heart weight/ Body weight (mg/gr)
K
Lung weight/ Body weight (mg/gr)
L
WT
iCox-2 KO
Female
Male
M
% Fibrosis
N
NT-pro-BNP (pg/ml)
WT
iCox-2 KO

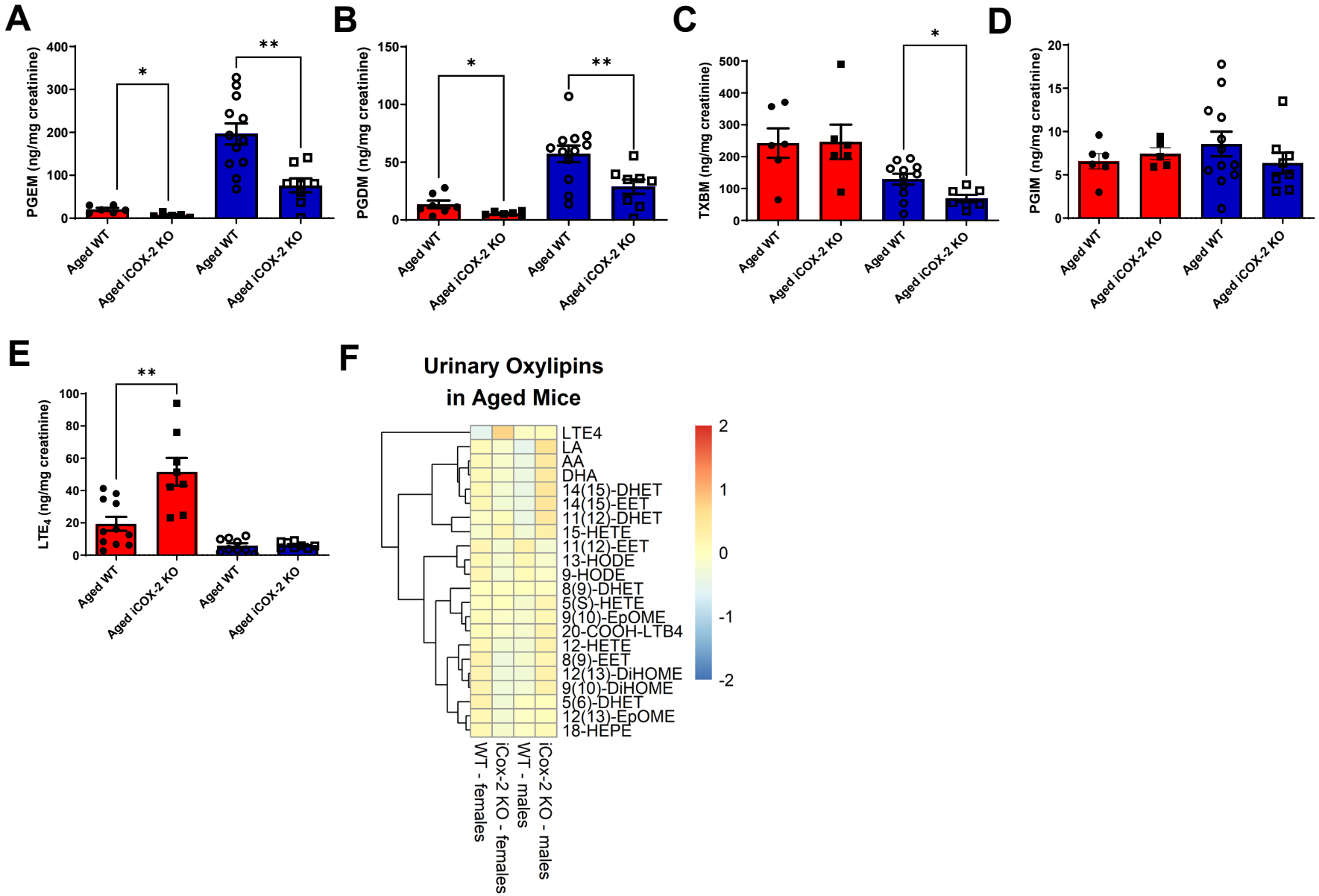
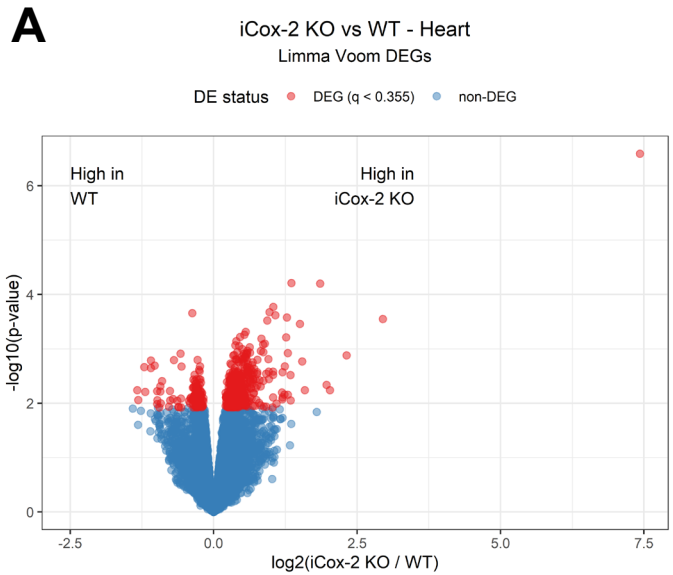
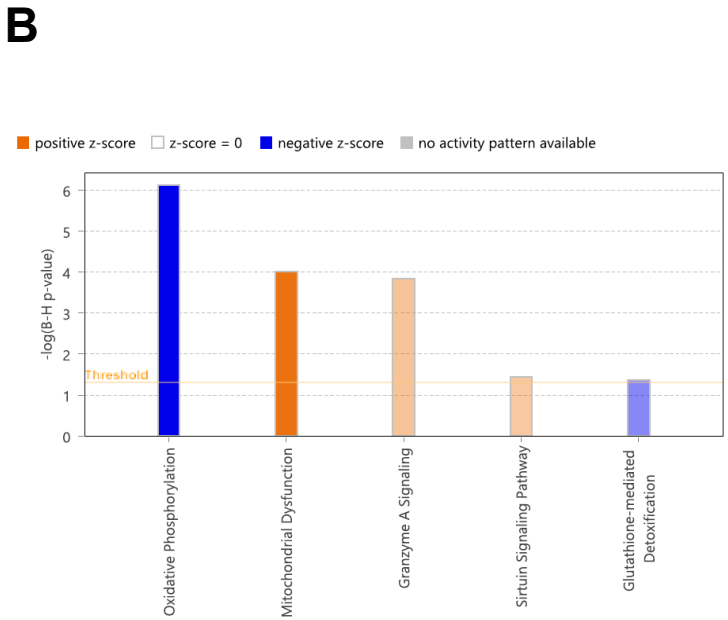

A
PGEM (ng/mg creatinine)
400
300
200
100
0
*
**
Aged WT
Aged iCOX-2 KO
Aged WT
Aged iCOX-2 KO
B
PGDM (ng/mg creatinine)
150
100
50
0
*
**
Aged WT
Aged iCOX-2 KO
Aged WT
Aged iCOX-2 KO
C
TXBM (ng/mg creatinine)
500
400
300
200
100
0
*
Aged WT
Aged iCOX-2 KO
Aged WT
Aged iCOX-2 KO
D
PGIM (ng/mg creatinine)
20
15
10
5
0
Aged WT
Aged iCOX-2 KO
Aged WT
Aged iCOX-2 KO
E
LTE4 (ng/mg creatinine)
100
80
60
40
20
0
**
Aged WT
Aged iCOX-2 KO
Aged WT
Aged iCOX-2 KO
F
Urinary Oxylipins
in Aged Mice
LTE4
LA
AA
DHA
14(15)-DHET
14(15)-EET
11(12)-DHET
15-HETE
11(12)-EET
13-HODE
9-HODE
8(9)-DHET
5(S)-HETE
9(10)-EpOME
20-COOH-LTB4
12-HETE
8(9)-EET
12(13)-DiHOME
9(10)-DiHOME
5(6)-DHET
12(13)-EpOME
18-HEPE
WT - females
iCox-2 KO - females
WT - males
iCox-2 KO - males
2
1
0
-1
-2

Fig. 2


A
iCox-2 KO vs WT - Heart
Limma Voom DEGs
DE status
DEG (q < 0.355)
non-DEG
High in WT
High in iCox-2 KO
-log10(p-value)
log2(iCox-2 KO / WT)


B
positive z-score
z-score = 0
negative z-score
no activity pattern available
-log(B-H p-value)
Threshold
Oxidative Phosphorylation
Mitochondrial Dysfunction
Granzyme A Signaling
Sirtuin Signaling Pathway
Glutathione-mediated Detoxification

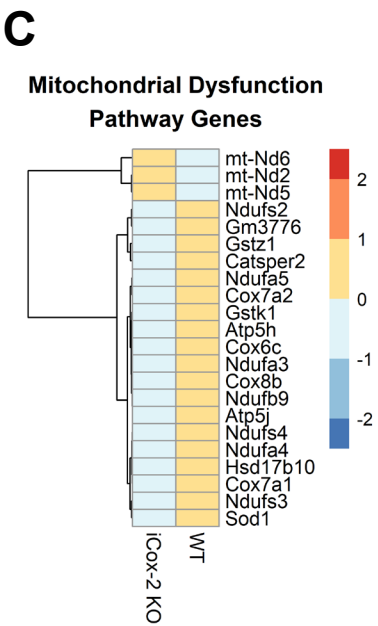

C
Mitochondrial Dysfunction Pathway Genes
mt-Nd6
mt-Nd2
mt-Nd5
Ndufs2
Gm3776
Gstz1
Catsper2
Ndufa5
Cox7a2
Gstk1
Atp5h
Cox6c
Ndufa3
Cox8b
Ndufb9
Atp5j
Ndufs4
Ndufa4
Hsd17b10
Cox7a1
Ndufs3
Sod1
iCox-2 KO
WT

Fig. 3

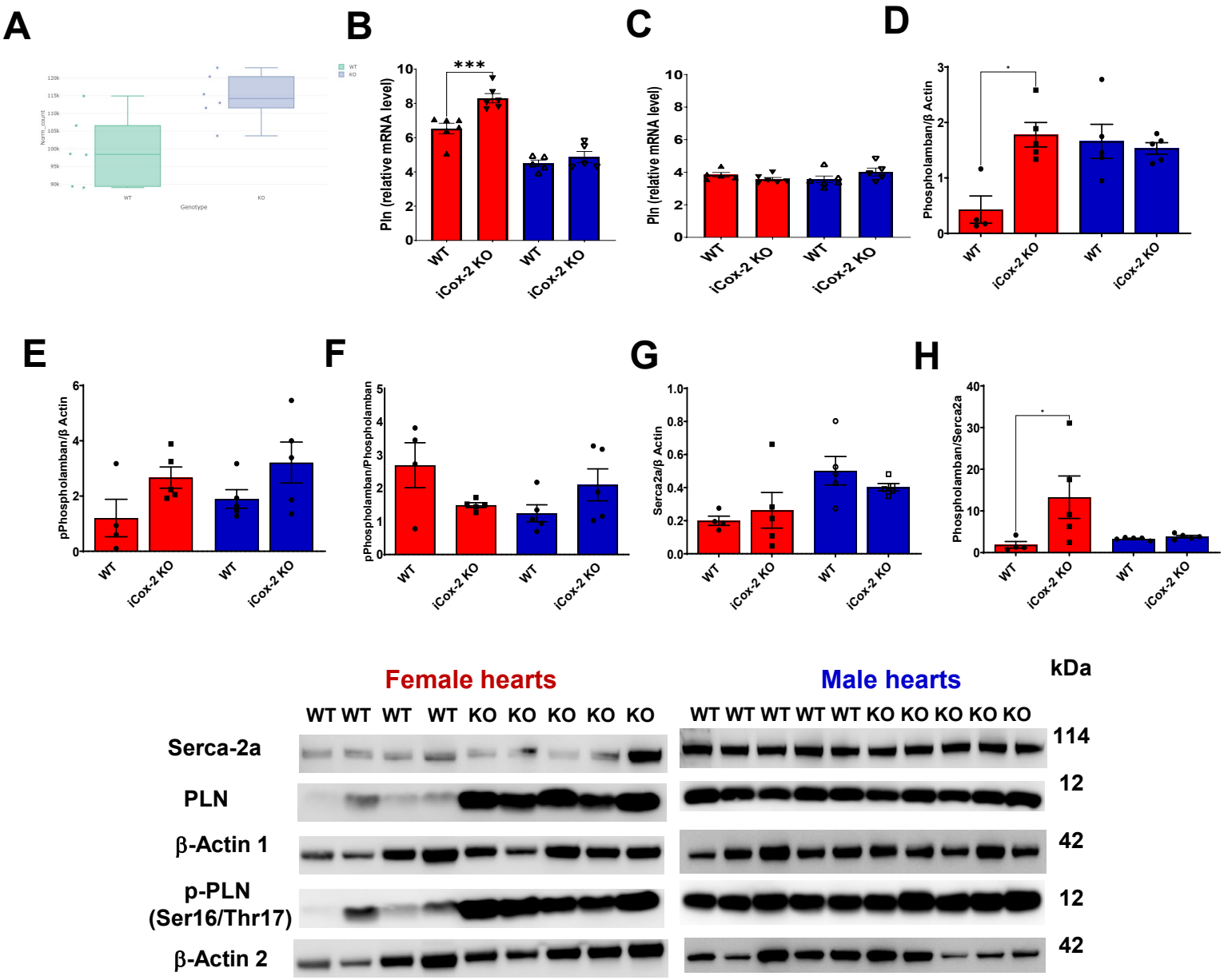

A
B
C
D
E
F
G
H
Pln (relative mRNA level)
Phospholamban/β Actin
pPhospholamban/β Actin
pPhospholamban/Phospholamban
Serca2a/β Actin
Phospholamban/Serca2a
WT
iCox-2 KO
***
Female hearts
Male hearts
kDa
WT WT WT WT KO KO KO KO KO
WT WT WT WT WT KO KO KO KO KO
Serca-2a
PLN
β-Actin 1
p-PLN
(Ser16/Thr17)
β-Actin 2
114
12
42
12
42

Fig. 4

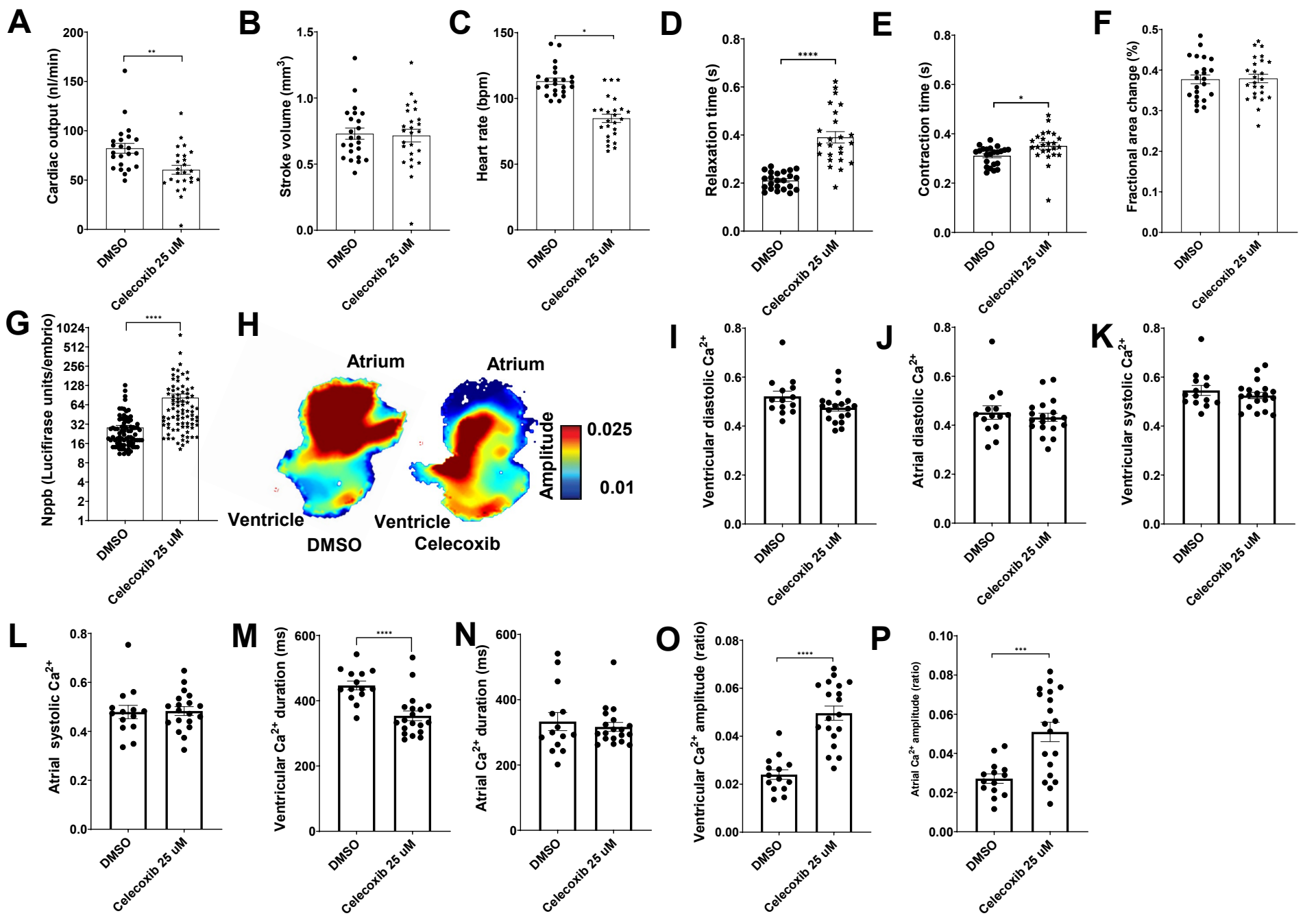
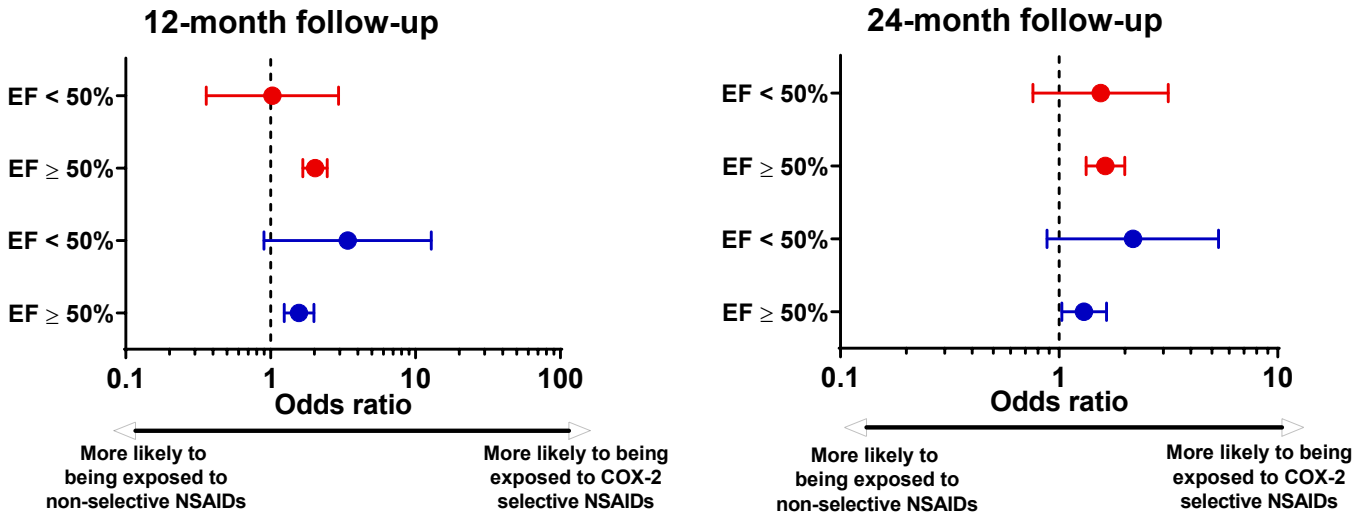

A
Cardiac output (nl/min)
B
Stroke volume (mm3)
C
Heart rate (bpm)
D
Relaxation time (s)
E
Contraction time (s)
F
Fractional area change (%)
G
Nppb (Lucifirase units/embrio)
H
Atrium
Atrium
Ventricle
Ventricle
DMSO
Celecoxib
Amplitude
0.025
0.01
I
Ventricular diastolic Ca2+
J
Atrial diastolic Ca2+
K
Ventricular systolic Ca2+
L
Atrial systolic Ca2+
M
Ventricular Ca2+ duration (ms)
N
Atrial Ca2+ duration (ms)
O
Ventricular Ca2+ amplitude (ratio)
P
Atrial Ca2+ amplitude (ratio)
DMSO
Celecoxib 25 uM

Fig. 5

A

No NSAID prescription before HF diagnosis



B

No NSAID and no diuretic prescription before HF diagnosis

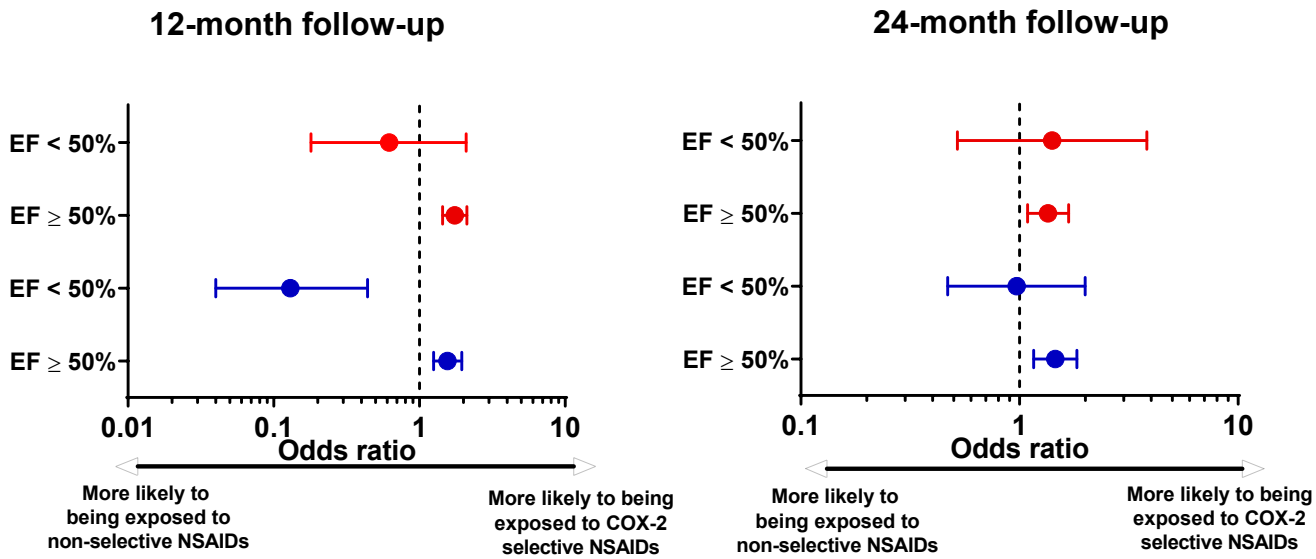
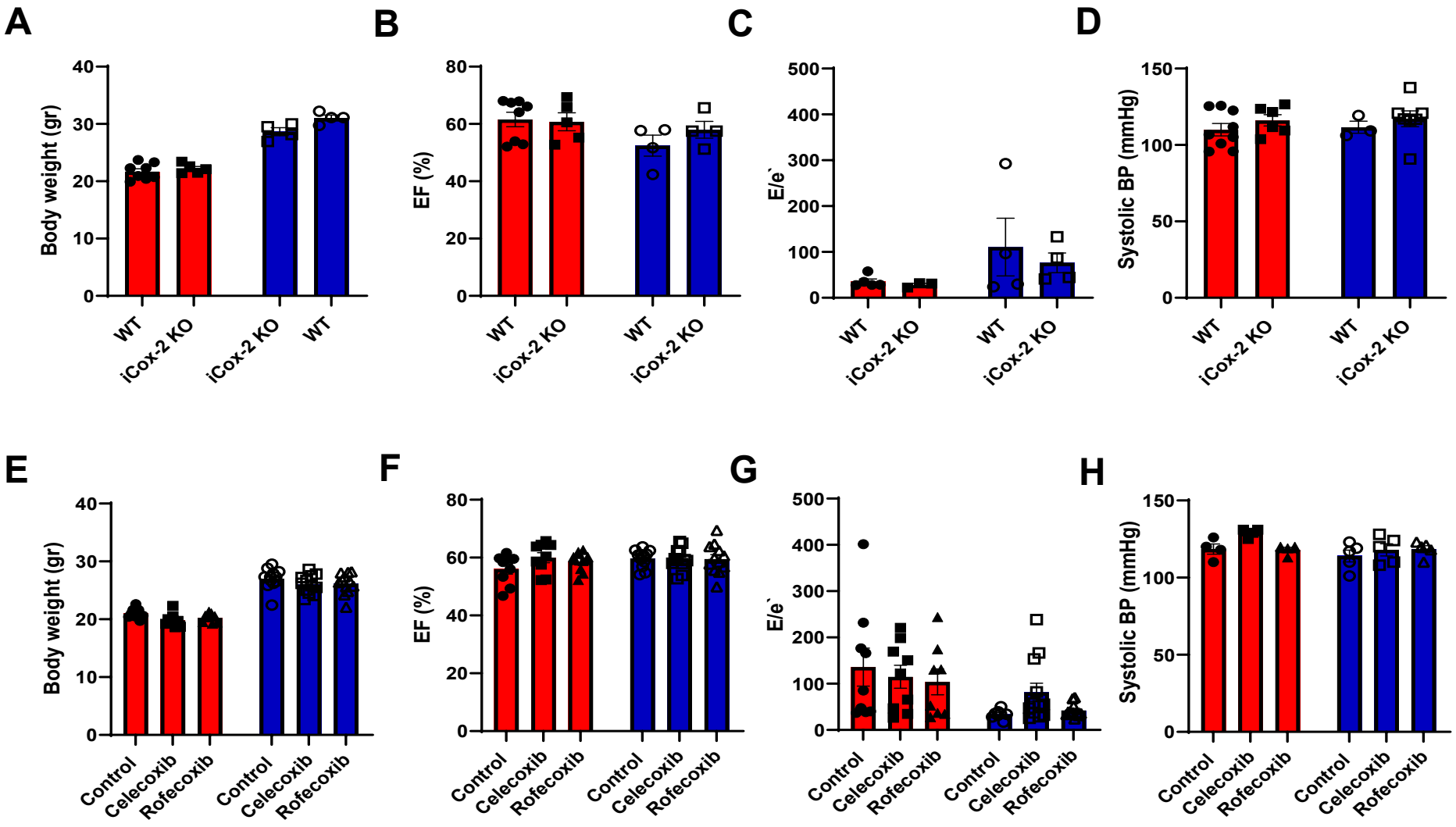
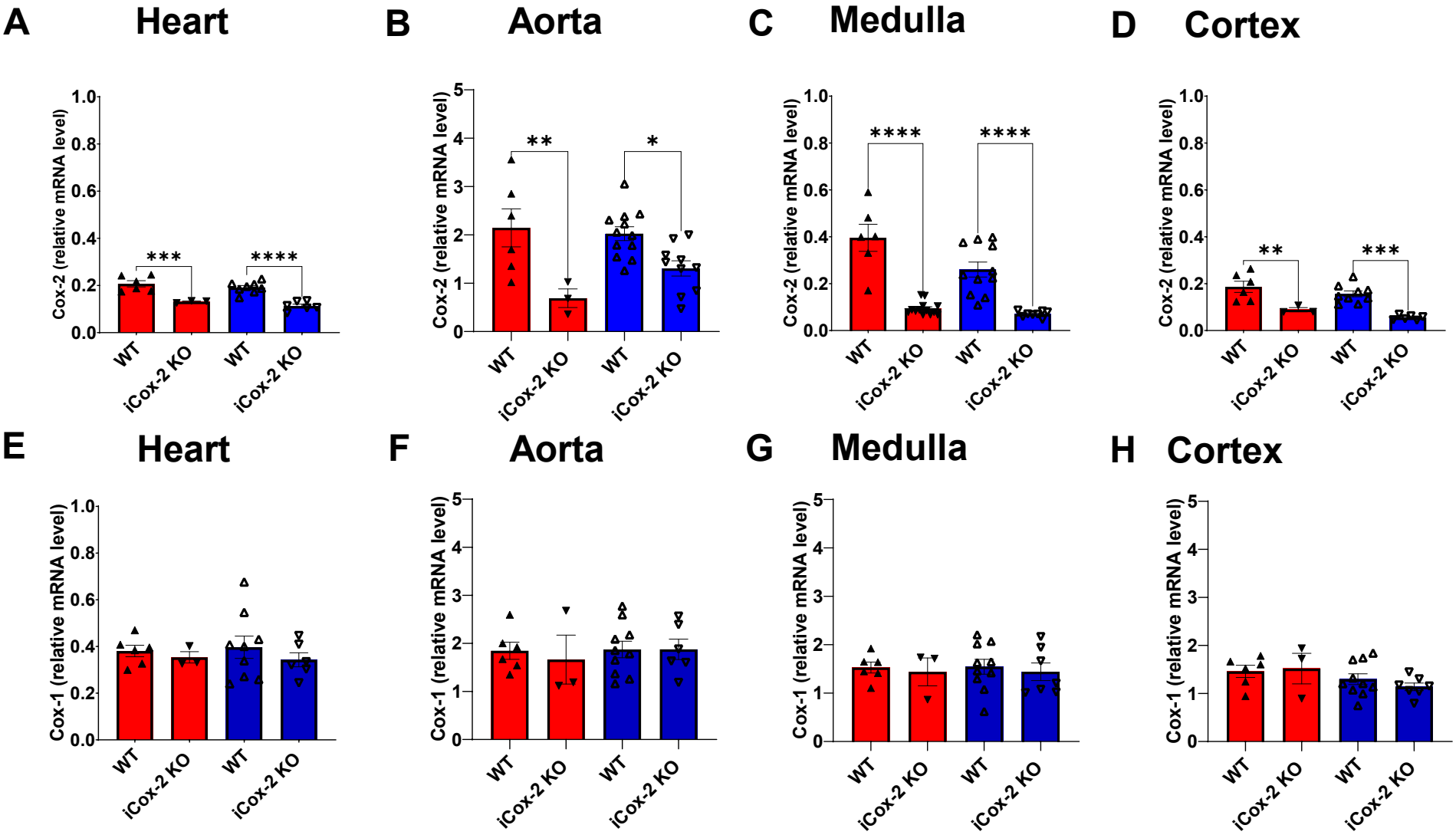


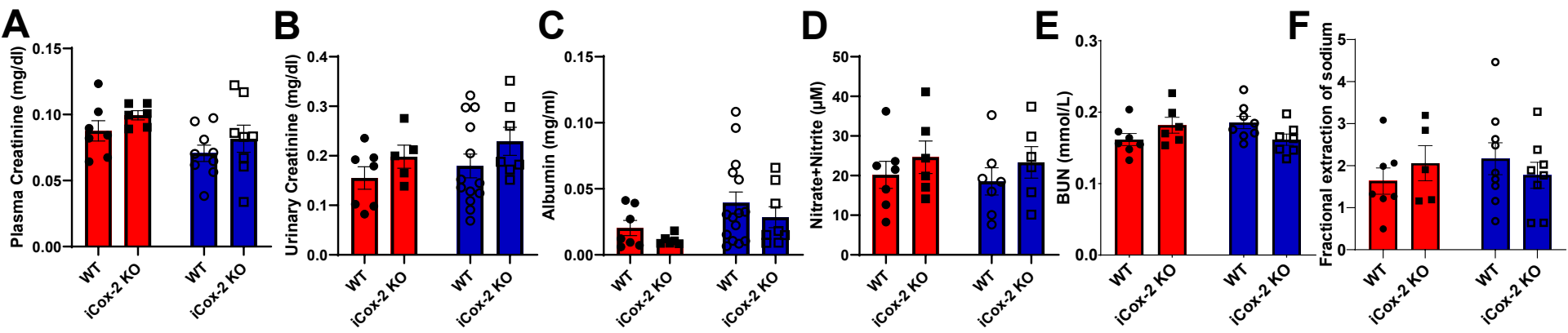
Fig. 6


A
Body weight (gr)
0
10
20
30
40
WT
iCox-2 KO
iCox-2 KO
WT
B
EF (%)
0
20
40
60
80
WT
iCox-2 KO
WT
iCox-2 KO
C
E/e`
0
100
200
300
400
500
WT
iCox-2 KO
WT
iCox-2 KO
D
Systolic BP (mmHg)
0
50
100
150
WT
iCox-2 KO
WT
iCox-2 KO
E
Body weight (gr)
0
10
20
30
40
Control
Celecoxib
Rofecoxib
Control
Celecoxib
Rofecoxib
F
EF (%)
0
20
40
60
80
Control
Celecoxib
Rofecoxib
Control
Celecoxib
Rofecoxib
G
E/e`
0
100
200
300
400
500
Control
Celecoxib
Rofecoxib
Control
Celecoxib
Rofecoxib
H
Systolic BP (mmHg)
0
50
100
150
Control
Celecoxib
Rofecoxib
Control
Celecoxib
Rofecoxib

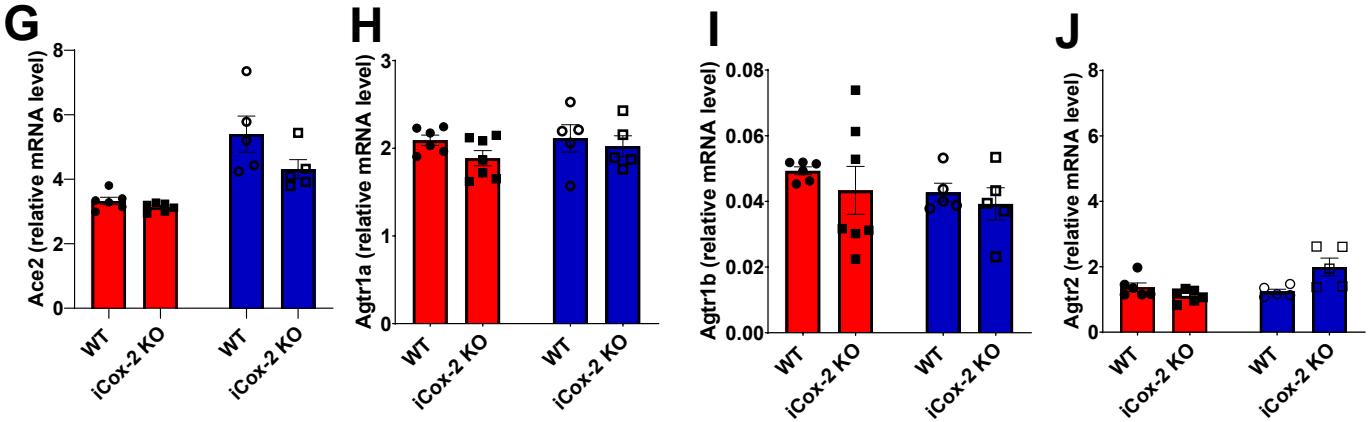
Suppl. Fig 1


A
Heart
B
Aorta
C
Medulla
D
Cortex
Cox-2 (relative mRNA level)
1.0
0.8
0.6
0.4
0.2
0.0
5
4
3
2
1
0
***
****
**
*
WT
iCox-2 KO
E
Heart
F
Aorta
G
Medulla
H
Cortex
Cox-1 (relative mRNA level)

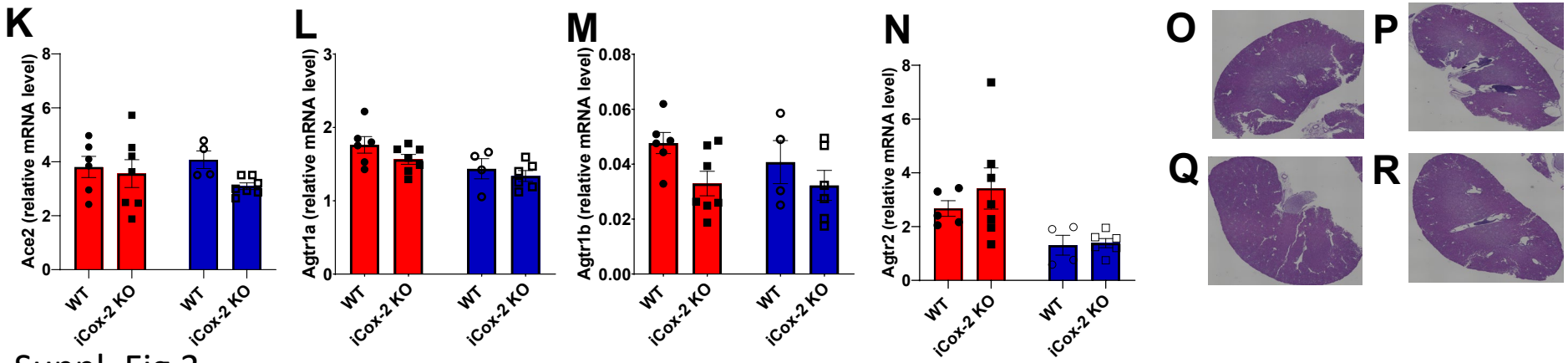
Suppl. Fig 2



Kidney cortex

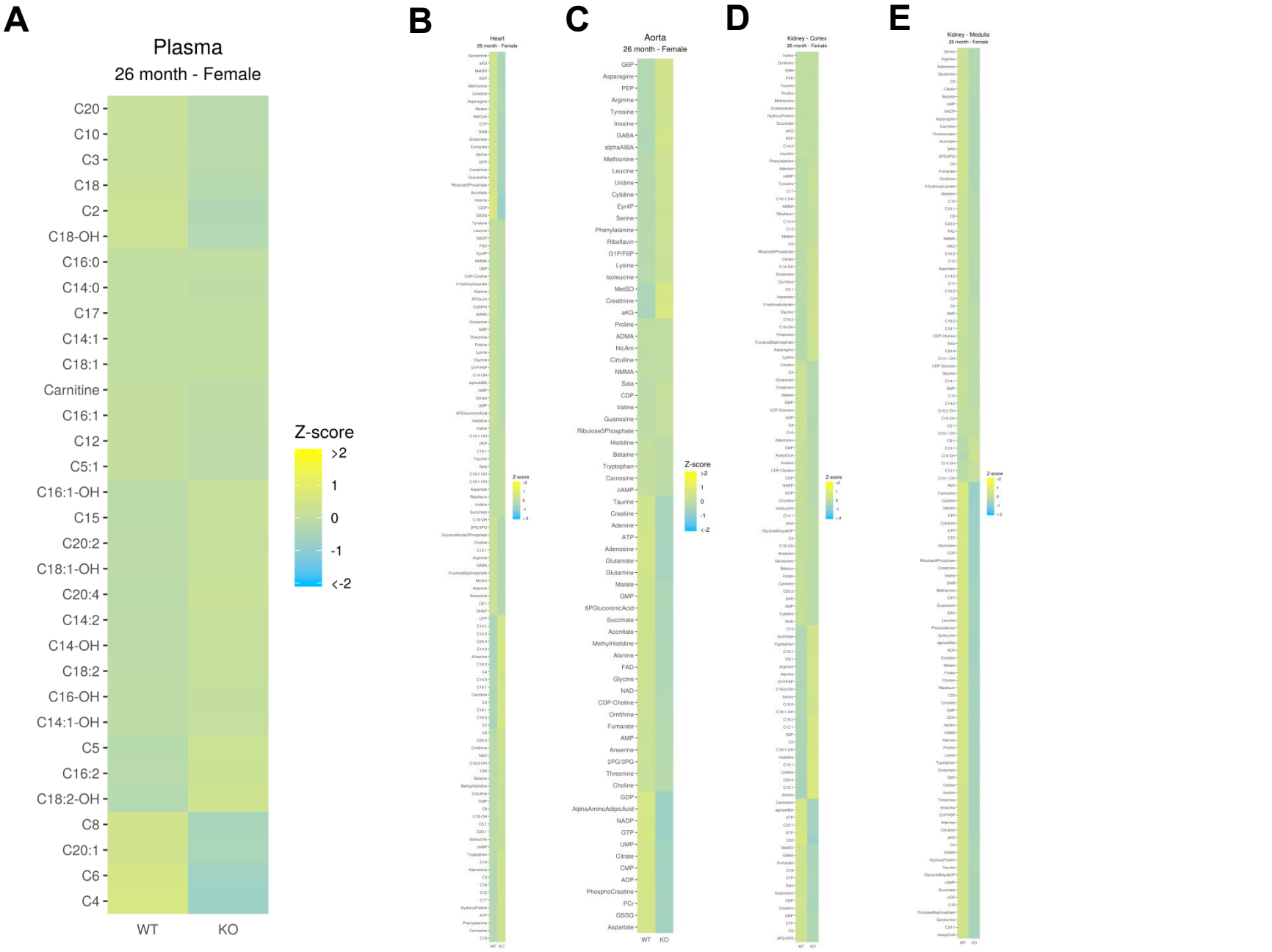


Kidney medulla

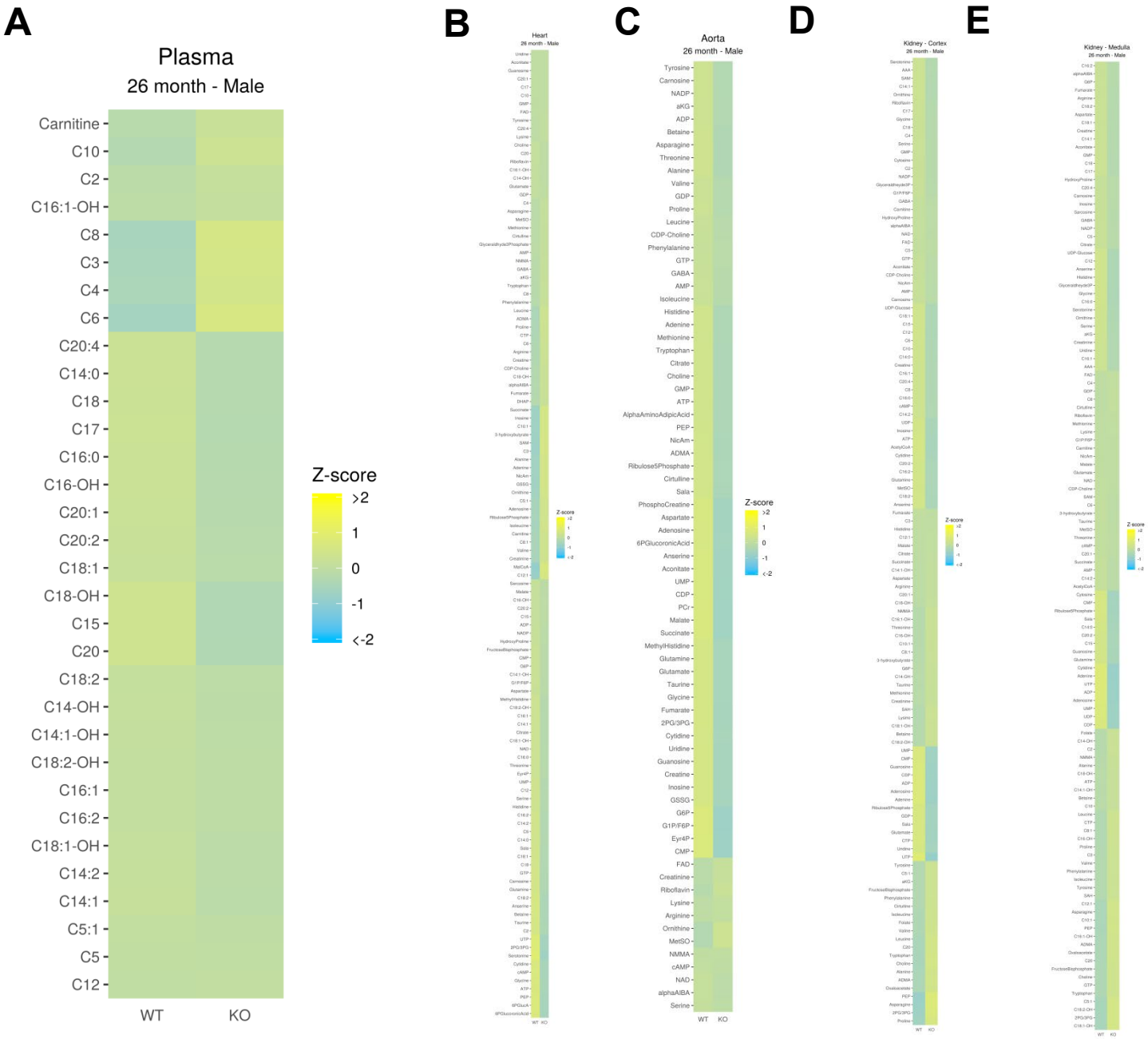


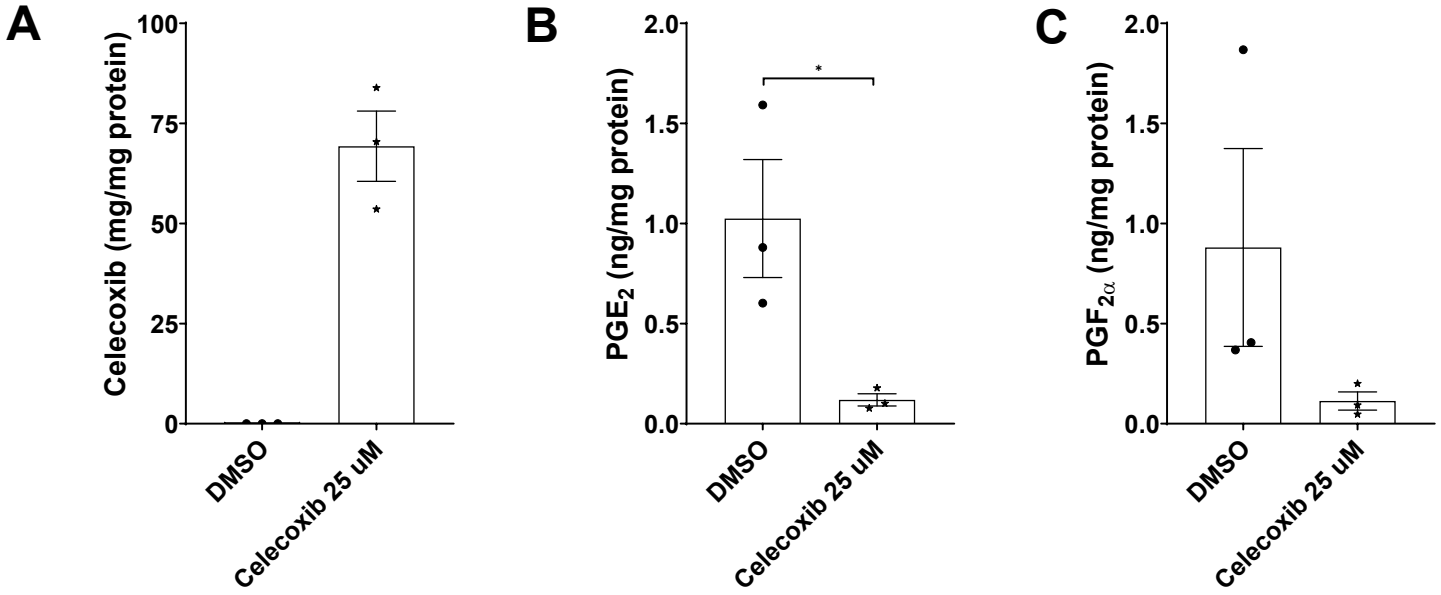
Suppl. Fig 3

Suppl. Fig 4


A
Plasma
26 month - Female
C20
C10
C3
C18
C2
C18-OH
C16:0
C14:0
C17
C14:1
C18:1
Carnitine
C16:1
C12
C5:1
C16:1-OH
C15
C20:2
C18:1-OH
C20:4
C14:2
C14-OH
C18:2
C16-OH
C14:1-OH
C5
C16:2
C18:2-OH
C8
C20:1
C6
C4
WT
KO
Z-score
>2
1
0
-1
<-2
B
Heart
26 month - Female
C
Aorta
26 month - Female
G6P
Asparagine
PEP
Arginine
Tyrosine
Inosine
GABA
alphaAIBA
Methionine
Leucine
Uridine
Cytidine
Ery4P
Serine
Phenylalanine
Riboflavin
G1P/F6P
Lysine
Isoleucine
MetSO
Creatinine
aKG
Proline
ADMA
NicAm
Citrulline
NMMA
Sala
CDP
Valine
Guanosine
Ribulose5Phosphate
Histidine
Betaine
Tryptophan
Carnosine
cAMP
Taurine
Creatine
Adenine
ATP
Adenosine
Glutamate
Glutamine
Malate
GMP
6PGlucoronicAcid
Succinate
Aconitate
MethylHistidine
Alanine
FAD
Glycine
NAD
CDP-Choline
Ornithine
Fumarate
AMP
Anserine
2PG/3PG
Threonine
Choline
GDP
AlphaAminoAdipicAcid
NADP
GTP
UMP
Citrate
CMP
ADP
PhosphoCreatine
PCr
GSSG
Aspartate
WT
KO
Z-score
>2
1
0
-1
<-2
D
Kidney - Cortex
26 month - Female
E
Kidney - Medulla
26 month - Female

Suppl. Fig 5


A
Plasma
26 month - Male
Carnitine
C10
C2
C16:1-OH
C8
C3
C4
C6
C20:4
C14:0
C18
C17
C16:0
C16-OH
C20:1
C20:2
C18:1
C18-OH
C15
C20
C18:2
C14-OH
C14:1-OH
C18:2-OH
C16:1
C16:2
C18:1-OH
C14:2
C14:1
C5:1
C5
C12
WT
KO
Z-score
>2
1
0
-1
<-2
B
Heart
26 month - Male
C
Aorta
26 month - Male
Tyrosine
Carnosine
NADP
aKG
ADP
Betaine
Asparagine
Threonine
Alanine
Valine
GDP
Proline
Leucine
CDP-Choline
Phenylalanine
GTP
GABA
AMP
Isoleucine
Histidine
Adenine
Methionine
Tryptophan
Citrate
Choline
GMP
ATP
AlphaAminoAdipicAcid
PEP
NicAm
ADMA
Ribulose5Phosphate
Cirtulline
Sala
PhosphoCreatine
Aspartate
Adenosine
6PGlucoronicAcid
Anserine
Aconitate
UMP
CDP
PCr
Malate
Succinate
MethylHistidine
Glutamine
Glutamate
Taurine
Glycine
Fumarate
2PG/3PG
Cytidine
Uridine
Guanosine
Creatine
Inosine
GSSG
G6P
G1P/F6P
Eyr4P
CMP
FAD
Creatinine
Riboflavin
Lysine
Arginine
Ornithine
MetSO
NMMA
cAMP
NAD
alphaAIBA
Serine
WT
KO
Z-score
>2
1
0
-1
<-2
D
Kidney - Cortex
26 month - Male
E
Kidney - Medulla
26 month - Male


A
Celecoxib (mg/mg protein)
100
75
50
25
0
DMSO
Celecoxib 25 uM
B
PGE2 (ng/mg protein)
2.0
1.5
1.0
0.5
0.0
*
DMSO
Celecoxib 25 uM
C
PGF2α (ng/mg protein)
2.0
1.5
1.0
0.5
0.0
DMSO
Celecoxib 25 uM

Suppl. Fig 6