

Blocking AMPK signalling to acetyl-CoA carboxylase increases cisplatin-induced acute kidney injury and suppresses the benefit of metformin

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ABSTRACT

Background: Acute kidney injury (AKI) is accompanied by dysregulation of cellular energy metabolism and accumulation of intracellular lipid. Phosphorylation of acetyl-CoA carboxylase (ACC) by AMP-activated protein kinase (AMPK) inhibits fatty acid synthesis and promotes fatty acid oxidation (FAO), vital for kidney tubular epithelial cells (TECs). The diabetes drug metformin is protective in models of AKI; however, it is not known whether ACC phosphorylation plays a role.

Methods: Cisplatin-induced AKI (CI-AKI) was established in ACC1/2 double knock-in (ACC1/2DKI) mice, harbouring mutations that disrupt fatty acid metabolism, and the role of metformin was studied in this model. Outcomes measured included serum biochemistry, expression of kidney injury markers such as neutrophil gelatinase-associated lipocalin (NGAL), and metabolomic analysis.

Findings: ACC1/2DKI mice demonstrated more severe CI-AKI than wild type (WT), as assessed by serum urea and creatinine, histological injury, and expression of NGAL and interleukin-6. Metformin protected against AKI in WT mice, shown by reduced NGAL, but this effect was absent in ACC1/2DKI mice. In cultured TECs exposed to cisplatin, metformin reduced expression of cleaved caspase-3, however, this effect was diminished in ACC1/2DKI TECs. Analysis of kidney polar metabolites found numerous differences between metformin-treated CI-AKI in ACC1/2DKI and WT mice, involving multiple pathways of amino acid, nucleoside, and energy metabolism.

Interpretation: Severity of CI-AKI is exacerbated by the inability to regulate metabolism via phosphorylation of ACC. ACC phosphorylation contributes to the protective effect of metformin against AKI, influencing multiple mechanisms involved in the pathogenesis of kidney injury.

1. Research in context

1.1. Evidence before this study

Fatty acid oxidation is a vital source of metabolic energy for kidney cells. Acute kidney injury is a major health problem characterised by reduced fatty acid oxidation and the accumulation of lipid in the kidney. Fatty acid oxidation is regulated by phosphorylation of acetyl-CoA

carboxylase (ACC) by the metabolic-sensor AMPK but the role of ACC phosphorylation in the acute kidney injury is unknown. The diabetes drug metformin activates AMPK and is protective against experimental kidney injury, however, the mechanisms for its protective effect in the kidney are not well understood.

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1.2. Added value of this study

In a model of cisplatin-induced kidney injury, this study found that phosphorylation of ACC by AMPK was protective against kidney injury, as mice with mutations of the ACC phosphorylation sites blocking AMPK signalling developed more severe disease. Furthermore, phosphorylation of ACC by AMPK contributed to metformin's protective effect, as shown by a loss of metformin's effect in the mice with the ACC mutations. Analysis of kidney metabolites found a role for multiple pathways of amino acid, nucleoside, and energy metabolism involved in the protective effects of metformin against cisplatin-induced kidney injury that were influenced by ACC phosphorylation.

1.3. Implications of all the available evidence

These data demonstrate the importance of changes in lipid metabolism in the development of kidney injury. AMPK mediated phosphorylation of ACC in response to AMPK activators such as metformin appears protective in acute kidney injury. The development of novel therapeutics to maintain renal fatty acid oxidation is a promising strategy for addressing the burdens of kidney disease.

2. Introduction

Acute kidney injury (AKI) is a major public health challenge, with more than 13 million cases globally per year [1] and associated with high mortality rate and the development of long-term chronic kidney disease [2]. An important cause of AKI is exposure to a wide range of nephrotoxins [3]. The kidney's susceptibility to toxin mediated injury relates to its physiologically high energy requirements, arising from its need for adenosine triphosphate to drive active transport across cell membranes [3]. Cisplatin-induced AKI (CI-AKI) is a limiting toxicity in cancer therapeutics, as well as being a widely employed experimental model for the study of nephrotoxic AKI [4].

β -oxidation of fatty acids (FAs) is a major energy source in the kidney, especially in the renal cortex and proximal tubular cells [5]. Dysregulation of energy metabolism and fatty acid oxidation (FAO) is a common feature of AKI arising from both nephrotoxic and ischaemic causes [6]. Furthermore, impaired FAO has been shown to be an important mechanism of renal fibrosis [7,8]. Acetyl-CoA carboxylase (ACC) is a rate limiting enzyme in the regulation of FAO and fatty acid (FA) synthesis [9]. It exists as two isoforms; ACC1 present in the cytosol and ACC2 localised to the outer mitochondrial membrane [9]. Inhibitory phosphorylation of ACC1 and ACC2 by AMP-activated protein kinase (AMPK) defends energy homeostasis by both increasing FAO and reducing FA synthesis [9].

The diabetes drug metformin protects against both acute and chronic kidney disease [10–12]. The benefits of metformin in AKI have been observed in diverse models, including with ischaemia [13], sepsis [14], and nephrotoxic AKI caused by cisplatin [15] or gentamicin [16]. In diabetes and metabolic diseases, indirect activation of AMPK has been identified as a mechanism central to metformin's therapeutic effects [17]. Phosphorylation of ACC by AMPK has been found to be required for the protective effect of metformin against renal fibrosis [8], however, its role in AKI is unknown.

In this study we aimed to determine the role of FA metabolism and ACC phosphorylation by AMPK in the outcome of CI-AKI, by studying the outcome in mice with knock-in Ser to Ala mutations of the ACC1-Ser⁷⁹ and ACC2-Ser²¹² phosphorylation sites. Furthermore, we used this model to determine the role of FA metabolism and ACC phosphorylation by AMPK in the protective effect of metformin in CI-AKI.

3. Methods

3.1. Animal studies

All animal experiments were approved by the Austin Health Animal Ethics Committee (Project number A2019_0565). Homozygous lines of ACC1/2 whole body double Ser to Ala knock-in (ACC1/2DKI) and wild type (WT) littermate control mice on a C57/Bl6 background were used, as previously described [8]. CI-AKI was established in male mice by intraperitoneal (i.p.) administration of cisplatin 20 mg/kg [15], given as two 10 mg/kg doses four hours apart, in mice 11–14 weeks old. In metformin experiments, metformin was administered 200 mg/kg i.p. for three consecutive days prior to CI-AKI, with control mice receiving vehicle (saline) injections. Outcomes were studied 2 days after the induction of AKI. Whole blood was collected by retro-orbital bleed, with serum sent to IDDEX laboratories (Vic, Aust) for measurement of urea and creatinine levels. Both kidneys were removed for tissue analysis.

3.1.1. Western blot analysis

Kidney lysates were prepared, and Western blots performed as previously described⁸. Quantification of Western Blots was by densitometry with analysis using Image J software [8], using glyceraldehyde 3-phosphate dehydrogenase (GAPDH) as a loading control. The following antibodies were used: neutrophil gelatinase associated lipocalin (NGAL) (Abcam, ab216462), GAPDH (Cell Signaling Technology 5174), cleaved caspase 3 (Cell Signaling Technology 9661), phospho-ACC (Abcam 68191) and carnitine palmitoyltransferase (CPT1) (Cell Signaling Technology 12252).

3.1.2. Histology

Kidney samples were fixed in 10% neutral buffered formalin (Sigma) and embedded in paraffin. Sections at 4 μ m thickness were used for periodic acid-Schiff (PAS) staining. To determine kidney injury, as defined by tubular necrosis, cellular casts, and tubular injury, a semi-quantitative scoring method was used, as previously described [15]. Score 0 represents injury area less than 10%, whereas score 1,2,3, or 4 represent the injury involving 10–25%, 25–50%, 50–75%, or > 75% of the field, respectively. At least ten randomly chosen fields under the microscope (\times 400) were evaluated for each mouse, and an average score was calculated.

3.1.3. Real-time polymerase chain reaction (qRT-PCR)

Total RNA was purified from whole mouse kidney samples and reverse transcribed as previously described [18]. RT-PCR was performed using the Livak method to calculate relative expression. Data was expressed as fold expression relative to littermate WT controls. Primer sequences are shown in [Supplementary Table 1](#).

3.1.4. Primary tubular epithelial cell cultures

Primary cultures of renal tubular epithelial cell cultures (TECs) were prepared by sieving whole mouse kidneys from female mice sacrificed at 4–7 weeks old, as previously described [8]. To mimic injury, cells were treated with 20 μ M cisplatin in serum-free K1 media (DMEM/F12, ITS, 25 mM HEPES, 100 U/ml penicillin, 100 μ g/ml streptomycin, 1 ng/ml prostaglandin, 5×10^{-11} M triiodothyronine, 5×10^{-8} M hydrocortisone, and 25 ng/ml mouse epidermal growth factor) for 6-hours or 23-hours. Cells were then lysed in cell lysis buffer (50 mM Tris/HCl, pH 7.5, 1 mM EGTA, 1 mM EDTA, 50 mM sodium fluoride, 5 mM sodium pyrophosphate, 1 mM sodium orthovanadate, 1% (w/v) NP-40, 0.27 M sucrose, 0.1% (v/v) 2-mercaptoethanol and 100 x protease inhibitor cocktail (Sigma P8340)) for protein analysis.

3.1.5. Metabolomics analysis

Gas Chromatography Mass Spectrometry (GC-MS) polar metabolomic analysis was performed, which targets for 350 polar metabolites, with broad representation across numerous metabolic pathways [19].

Whole kidney samples were extracted in a cryogenic bead mill with 1 ml 3:1 MeOH:H₂O (v/v) containing ¹³C,¹⁵N-valine and ¹³C-sorbitol internal standards. Homogenate (800 µl) was transferred to fresh tubes and 200 µl chloroform added to a ratio of 1:3:1 CHCl₃:MeOH:H₂O (v/v/v). The mixture was allowed to extract for 10 min at 4°C, then centrifuged to pellet precipitated protein and cell debris. A volume of supernatant, relative to kidney sample weight, was dried down for GC-MS analysis. Pooled Biological Quality Controls (PBQCs) were generated by pooling an aliquot (30 µl) of each sample. 50 µl of this pooled supernatant was aliquoted into 7 replicates for analysis throughout the sequence. Samples were run in a randomised order to avoid sequential bias. Samples and PBQCs were methoximated and trimethylsilylated to enable gas phase analysis. 1 µl of derivatised sample was analysed on a Shimadzu GC-TQ8050NX using the Shimadzu Smart Metabolite Database. (10:1 split injection). Biological samples were run in a randomised order, with PBQCs interspersed every 5 samples. Raw data files were processed using Shimadzu Labsolutions Insight software. Internal standard coefficients of variation were calculated for PBQCs only, to confirm good data quality (a CV of <20%). The cleaned data matrix was imported into Metaboanalyst 5.0 (metaboanalyst.ca) for statistical analyses. Data was log-transformed and median-normalised prior to performing PCA and Heatmap analysis. Data quality was further determined by observation of PBQC clustering.

3.1.6. Statistics

Statistical analyses were performed using Prism version 7.0a for MacOS X (GraphPad Software, San Diego, CA). Data are presented as mean ±SD. RT-PCR data are presented as mean±SEM. Multiple group means were compared by ANOVA followed by a post hoc test. Comparison of means from two groups was performed by an unpaired t test. P values of < 0.05 were considered significant.

Statistical analysis of kidney polar metabolite profiles was performed using Metaboanalyst 5.0.

3.1.7. Role of the funding source

The funders played no part in the design, data analyses, interpretation, writing of report, or decision to publish the results.

4. Results

4.1. The effect of ACC phosphorylation on acute kidney injury

CI-AKI was established in male ACC1/2DKI and WT mice. ACC1/2DKI mice exhibited more severe AKI, as assessed by day 2 serum urea (ACC1/2DKI 40.5 ±11.6 mM vs WT 27.2 ±7.6 mM, p = 0.0013) (Fig. 1A) and creatinine (ACC1/2DKI 0.09 ±0.03 mM vs WT 0.06 ±0.03 mM, p = 0.021) (Fig. 1B). Western blot for the injury marker NGAL also revealed greater injury in ACC1/2DKI mice (Fig. 1C), with a 9.3 ± 2.1-fold increase in ACC1/2DKI compared to 3.3 ± 3.4-fold in WT (p <

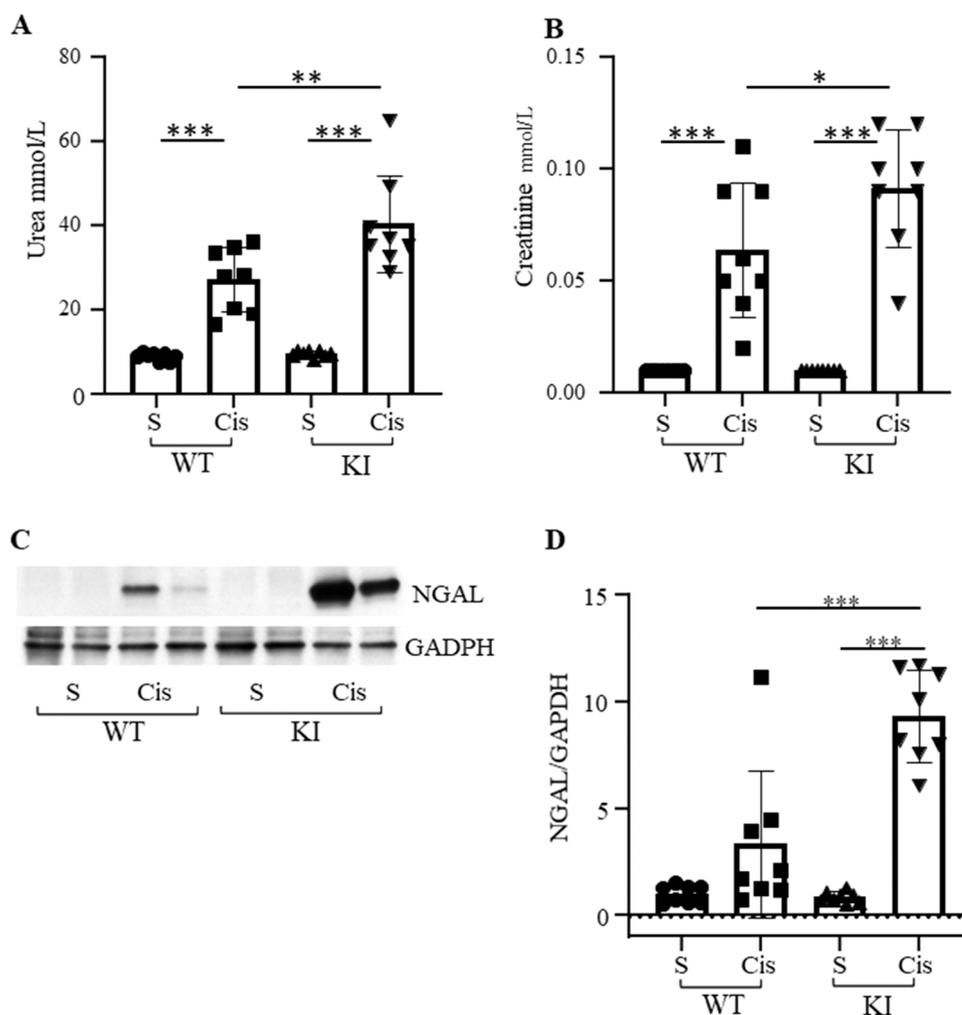


Fig. 1. Outcome of CI-AKI in ACC1/2DKI (KI) and WT mice. Mice were injected i.p. with cisplatin (Cis) or saline (S) (N = 8 per group) and outcomes were analyzed after 48 h. 1A: Serum urea. 1B Serum creatinine. 1C. NGAL expression from whole kidney homogenate by Western blot. 1D. NGAL expression quantified by densitometry analysis of Western blots (N = 8), normalised for GAPDH. All results are shown as mean ± SD. * p < 0.05, ** p < 0.01, *** p < 0.001.

0.0001 for Cis-ACC1/2DKI vs Cis-WT) (Fig. 1D). After 2 days, weight loss in the mice was not different between ACC1/2DKI (11.5 ± 1.3%) and WT (12.7 ± 1.3%) mice. Together, these data indicate that blocking AMPK signalling to ACC in ACC1/2DKI mice, exacerbates the severity of nephrotoxic AKI caused by cisplatin.

Histological analysis (PAS stain) of kidney injury revealed widespread signs of injury in ACC1/2DKI and WT mice, with features including tubular necrosis, tubular dilatation, tubular vacuolation, and cast formation (Fig. 2B, D). Consistent with the biochemical and NGAL data, quantification of the histological injury score demonstrated more severe AKI in ACC1/2DKI mice as compared to WT (injury score 3.1 ± 0.34 vs 2.5 ± 0.63, $p = 0.022$) (Fig. 2E).

4.1.1. Effects of cisplatin-induced AKI on measures of metabolism

CI-AKI was associated with reduced expression of phosphorylated ACC, suggesting a shift to increased FA synthesis and reduced FAO (Fig. 3A, 3C). As expected, ACC phosphorylation was not detectable in ACC1/2DKI mice. The reduced expression of phosphorylated ACC observed here with CI-AKI is compatible with previous studies showing reduced AMPK activity and ACC phosphorylation with cisplatin exposure [20–22]. CPT1, which translocate fatty acids from the cytosol to the mitochondrial matrix, was significantly reduced with CI-AKI in both WT

and ACC1/2DKI mice as analysed by both Western blot (3B, 3D) and RT-PCT (3E). Similarly, peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC-1 α), which regulates mitochondrial biogenesis, was reduced with CI-AKI in both WT and ACC1/2DKI strains (Fig. 3G). Unexpectedly, PGC1 α expression was increased in saline treated ACC1/2DKI mice compared with WT, although we note that this finding was not observed in an earlier study [8], raising the possibility of a type 1 error. Acyl-CoA oxidase-1 (Acox1), a rate-limiting enzyme in peroxisomal fatty acid β -oxidation, was also reduced with AKI, although this was only significant in ACC1/2DKI mice (Fig. 3F). Cytochrome c oxidase subunit 4 (Cox4), the last enzyme in the respiratory electron transport chain located in the mitochondrial membrane, was reduced with AKI in the ACC1/2DKI mice but not with WT (Fig. 3H). Citrate synthase (CS), a citric acid cycle enzyme, was non-significantly reduced with AKI (Fig. 3I). Overall, these data suggest that a difference in mitochondrial abundance is unlikely to explain the more severe AKI phenotype observed in the ACC1/2DKI mice.

4.1.2. Effects of cisplatin-induced AKI on measures of inflammation

Activation of inflammation pathways correlates with injury in CI-AKI; therefore, markers of inflammation were measured by RT-PCR. IL-1 increased 2.5 ± 0.40-fold ($p < 0.001$) with AKI in ACC1/2DKI

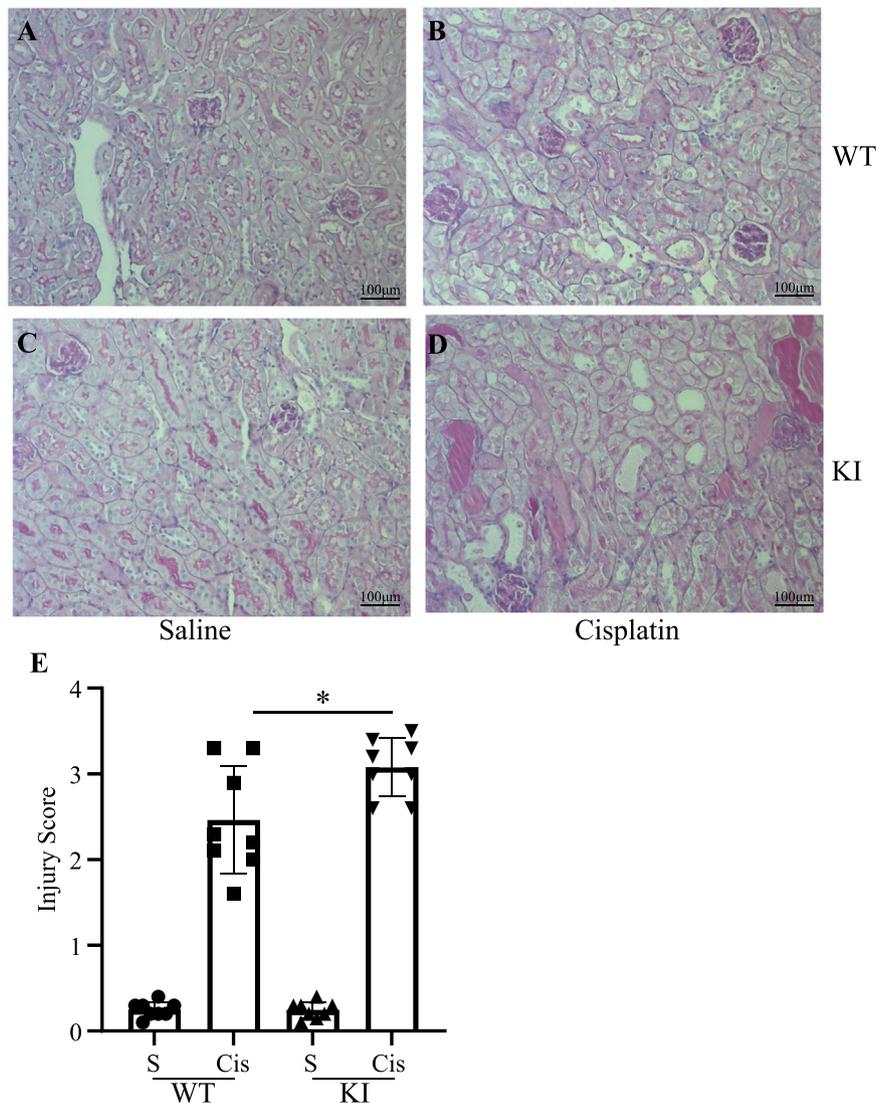


Fig. 2. : Histological kidney injury after CI-AKI in ACC1/2DKI (KI) and WT mice analysed by PAS stain. 2A: WT – saline treated. 2B: WT – cisplatin treated. 2C: ACC1/2DKI – saline treated. 2D: ACC1/2DKI – cisplatin treated. Images were taken at 200x magnification. Scale bar of 100 μm is shown. 2E: Histological injury score * $p = 0.022$.

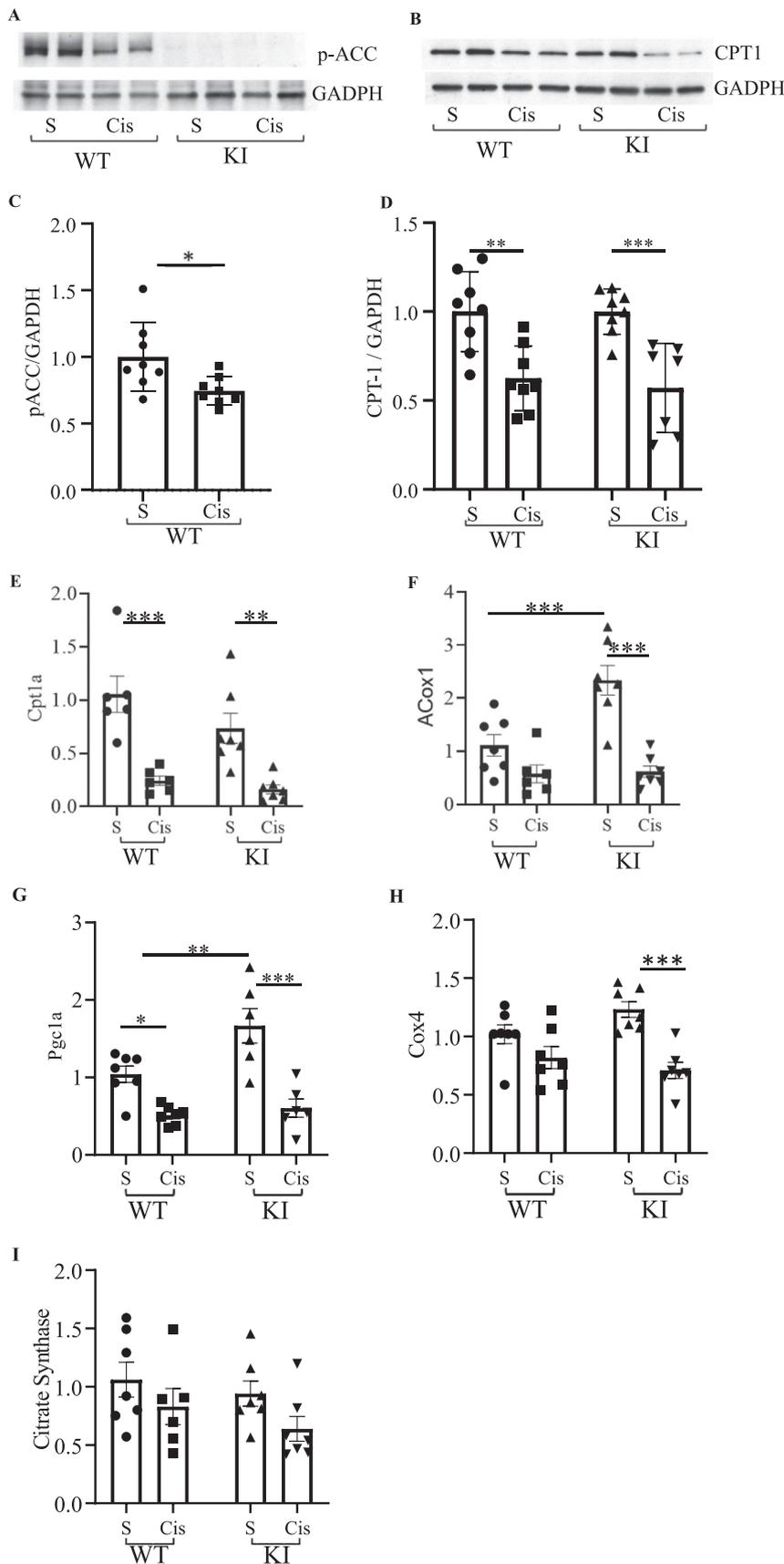


Fig. 3. : Effects of CI-AKI on markers of metabolism. 3 A. Western blot showing ACC phosphorylation with CI-AKI in WT and ACC1/2DKI (KI) mice. 3 C. Densitometric quantification of ACC phosphorylation, normalised for GAPDH. 3B. Western blot for CPT1 expression. 3D. Densitometric quantification of CPT1 expression, normalised for GAPDH. ³E-³I: Expression of energy metabolism genes quantified by RT-PCR (3E: Cpt1a, 3 F: AcCo1, 3 G: Pgc1a, 3 H: Cox4, 3I: citrate synthase. *p < 0.05, **p < 0.01, ***p < 0.001.

mice but was not increased with AKI in WT mice (Fig. 4A). IL-6 increased with AKI, with a significantly greater increase in ACC1/2DKI mice 31.3 ± 5.7-fold compared to WT mice 11.4 ± 0.95-fold ($p = 0.0004$ for ACC1/2DKI vs WT) (Fig. 4B). VCAM was significantly increased in ACC1/2DKI mice (2.3-fold, $p = 0.01$) but not in WT mice ($p = 0.06$) (Fig. 4E). ICAM and TNF α were significantly increased with AKI, but not different between WT and ACC1/2DKI (Fig. 4D, F). These data indicate that differential expression of IL-1 and IL-6 may contribute to the difference observed in CI-AKI severity between WT and ACC1/2DKI mice.

4.1.2.1. Polar Metabolites Analysis of CI-AKI in WT and ACC1/2DKI. To further understand the effect of CI-AKI on kidney metabolism in WT and ACC1/2DKI mice we performed polar metabolite analysis by GC-MS, which revealed marked changes in both WT and ACC1/2DKI strains (Fig. 5). Volcano plot analysis found that CI-AKI altered 89/220 (40.5%) detectable polar metabolites in WT and 98/220 (44.6%) (thresholds for difference: fold change (FC) > 1.3, FDR $P < 0.05$) (Supp_Figs. 1A,1B).

Heatmap analysis of the 50 most altered polar metabolites at day 2 CI-AKI is shown in Fig. 5 A, with dendrogram analysis showing separation of the WT and ACC1/2DKI stains in the cisplatin treated animals. These data indicate disruption of multiple metabolic pathways including those involved in energy generation, and amino acid and lipid metabolism. A notable finding with CI-AKI was disruption of the kynurenine pathway, which accounts for production of NAD $^+$, an important protective factor against AKI [23] (Supp_Fig. 1 C). Regarding the kynurenine pathway, CI-AKI resulted in markedly increased levels of the upstream metabolites quinolinic acid and kynurenic acid, but depletion of the downstream metabolites, nicotinic acid and niacinamide, consistent with a block at the level of the bottleneck enzyme quinolate phosphoribosyl transferase (QPRT) leading to reduced availability of NAD $^+$. RT-PCR analysis confirmed that QPRT was markedly reduced by CI-AKI, although, there was no difference between the WT and ACC1/2DKI genotypes (Fig. 5B).

A notable feature of CI-AKI was lower levels of multiple purine and pyrimidine nucleosides, including adenosine, guanosine, inosine, and

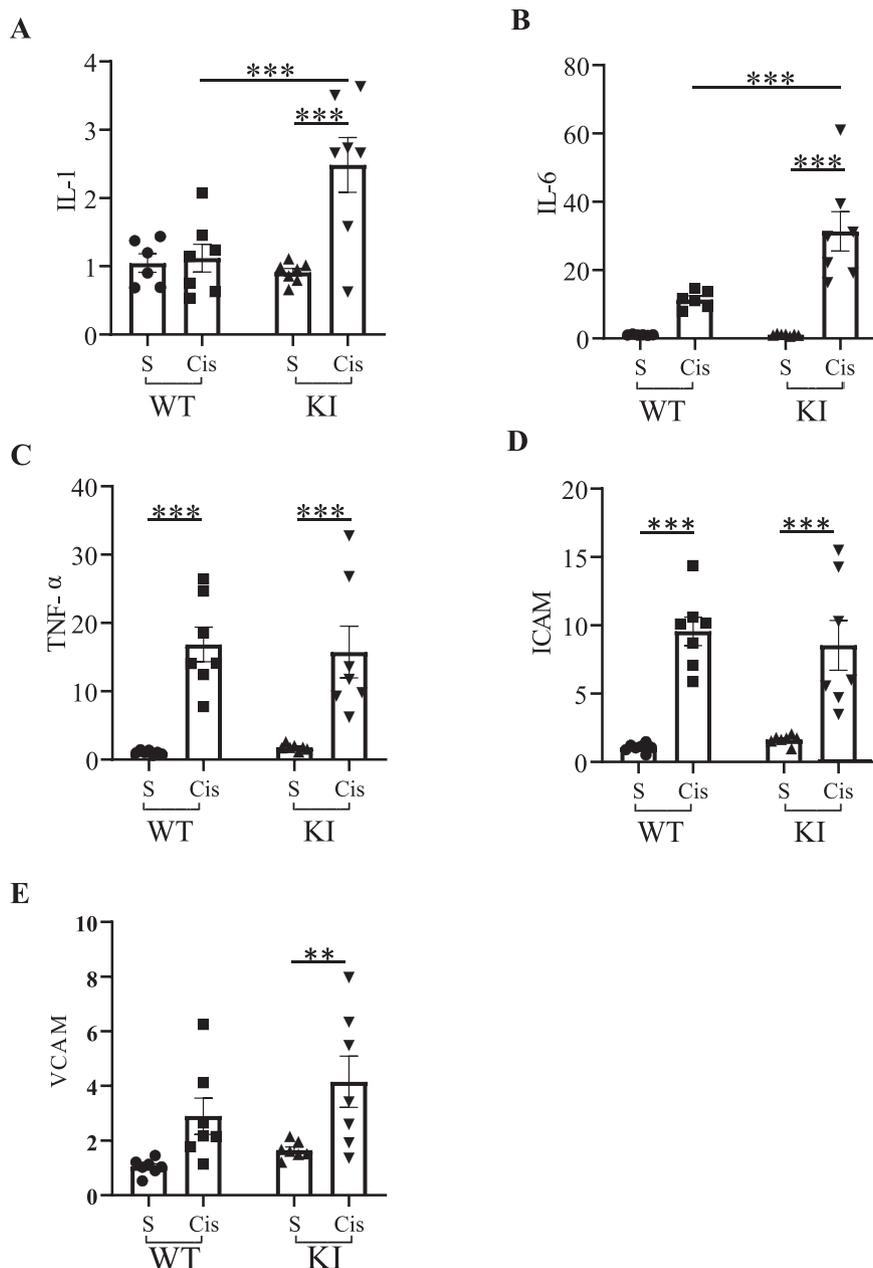


Fig. 4. : Effects of CI-AKI on markers of inflammation. 4A-E: Expression of inflammation genes quantified by RT-PCR (4 A: IL-1, 4B: IL-6, 4 C: TNF α , 4D: ICAM, 4E: VCAM). ** $p = 0.01$, *** $p < 0.001$.

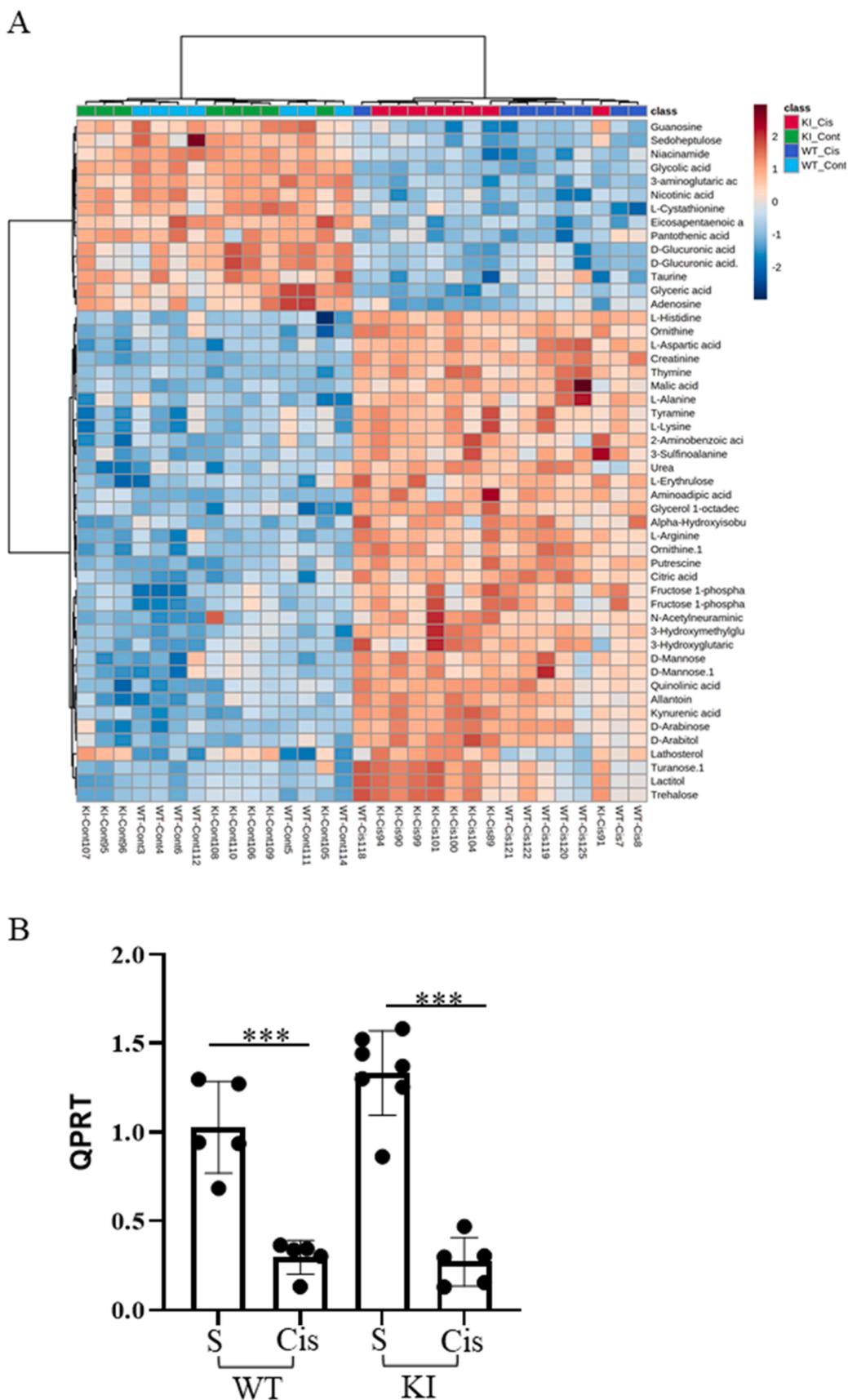


Fig. 5. 5A. Heatmap analysis of the 50 most altered kidney metabolites comparing WT-control, WT-CI-AKI, ACC1/2DKI-control, and ACC1/2DKI-CI-AKI groups. 5B. Expression of QPRT by RT-PCR *** $p < 0.001$.

uridine, although this was not different between ACC1/2DKI and WT. With regards to lipids, the cholesterol intermediate lathosterol was increased in ACC1/2DKI mice as compared to WT (Fig. 5 A). Interestingly, levels of saturated FAs such as myristic acid and palmitic acid did not differ between groups at day 2 CI-AKI. The omega 3 polyunsaturated acids eicosapentaenoic acid and docosahexaenoic acid were reduced by CI-AKI but did not differ between ACC1/2DKI and WT.

4.1.2.2. The role of ACC phosphorylation in the protective effect of metformin in acute kidney injury. Previous studies have shown that pharmacological activation of AMPK by metformin and other activators protects against CI-AKI [15,21,24], however, the contribution of ACC phosphorylation by AMPK to this effect of metformin has not been studied. To address this question, we treated WT and ACC1/2DKI mice with metformin, prior to the induction of CI-AKI. In WT mice given cisplatin, metformin protected against AKI at day 2, as evidenced.

by lower serum urea (WT-saline 35.4 +4.4 mM vs WT-metformin 22.4 +6.1 mM) (Fig. 6A) and creatinine (WT-saline 0.08 +0.01 mM vs

WT-metformin 0.04 +0.01 mM) (Fig. 6B). In ACC1/2DKI mice, metformin did not protect against AKI, with no difference in either serum urea (ACC1/2DKI-saline 56.8 +23.7 mM vs ACC1/2DKI-metformin 51.2 +32.2 mM) (Fig. 6A) or creatinine (WT-saline 0.11 +0.05 mM vs WT-metformin 0.11 +0.08 mM) (Fig. 6B). When comparing the metformin treated mice, both serum urea (WT-metformin 22.4 +6.1 mM vs ACC1/2DKI-metformin 51.2 +32.2 mM, $p = 0.009$) and creatinine (WT-metformin 0.04 +0.01 mM vs ACC1/2DKI-metformin 0.11 +0.08 mM, $p = 0.014$) were elevated in ACC1/2DKI mice as compared to WT (Fig. 6A,B). The severity of AKI was also assessed by Western blot for NGAL expression which was reduced by 51% with metformin treatment in WT mice ($p = 0.003$), however, in ACC1/2DKI mice no difference with metformin therapy was observed (Fig. 6C, D).

With regards to ACC, Western blot analysis for total ACC found no differences between saline and metformin, or between WT and ACC1/2DKI (6E), a finding consistent with a previous study [8]. ACC phosphorylation was absent in ACC1/2DKI mice, and post Cis-AKI was not different between saline and metformin in WT mice (6E, 6 F). Metformin

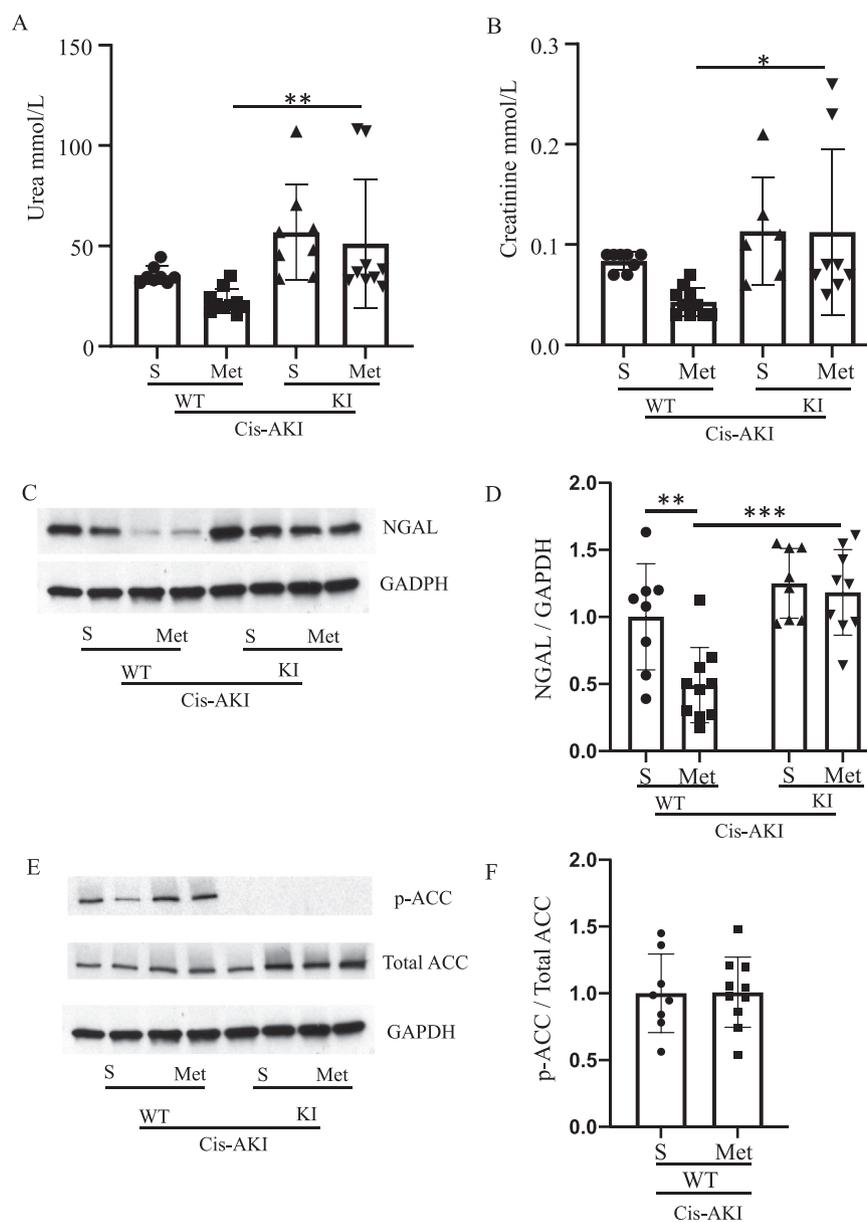


Fig. 6. : The effect of metformin on CI-AKI in WT and ACC1/2DKI mice (N = 8–10 per group). 6 A. Serum urea. 6B. Serum creatinine. 6 C. NGAL expression from kidney homogenate by Western blot. 6D. NGAL expression quantified by densitometry analysis of Western blots normalized for GAPDH. 6E. p-ACC and Total ACC expression from kidney homogenate by Western blot. 6 F. p-ACC expression quantified by densitometry analysis of Western blots normalized for Total ACC.

is known to increase ACC phosphorylation via AMPK, and previous studies have observed increased ACC phosphorylation in the kidney after treatment with metformin [8,25]. The absence of a metformin effect on ACC phosphorylation in the present study may reflect that the timing of the analysis was performed 48-hours after the onset of Cis-AKI, which is known to reduce ACC phosphorylation, and 72-hours after the completion of metformin pre-treatment.

Consistent with the biochemical and NGAL data, histological analysis by PAS stain found that metformin reduced the injury score in WT mice but not in KI mice. (Fig. 7).

The role of ACC phosphorylation in metformin's effect on cisplatin-mediated cellular injury was further assessed in primary TECs from female ACC1/2DKI and WT mice (Fig. 8). Western blot for cleaved caspase 3 was selected as a marker of cisplatin mediated cellular injury based on previous studies [15,24]. Consistent with these previous studies, we observed that cleaved caspase 3 expression was not detected in untreated TECs, but clearly detectable by Western blot after cisplatin exposure. Incubation in 4 mM metformin for 4-hours prior to 20 μ M cisplatin exposure for 23 h reduced cleaved caspase 3 expression by 72%

in WT cells versus 49% in ACC1/2DKI cells ($p = 0.03$ for difference between WT and ACC1/2DKI) (Fig. 8B). This analysis was performed using a cleaved caspase-3 antibody (Cell Signaling Technology 9661), which detects the large fragment (17/19 kDa) of activated caspase-3 resulting from cleavage adjacent to Asp175. This antibody does not recognize full length caspase-3 or other cleaved caspases. Regarding the significance of increased expression of cleaved caspase 3, this has been most commonly associated with apoptosis, however, caspase 3 involvement with other processes such as autophagy, cellular differentiation, cellular proliferation, and tumorigenesis, has also been described [26]. Taken together, these data from the whole animal and cell culture studies indicate that AMPK mediated phosphorylation of ACC contributes to the protective effect of metformin against CI-AKI.

4.1.2.3. Analysis of the Metformin Effect on Polar Metabolites in CI-AKI in ACC1/2DKI Mice. To further understand the effects of ACC phosphorylation and the action of metformin on cisplatin-AKI we performed analysis of polar metabolites by GC-MS. When comparing kidneys of saline treated ACC1/2DKI and WT mice only 1/217 (0.46%) of

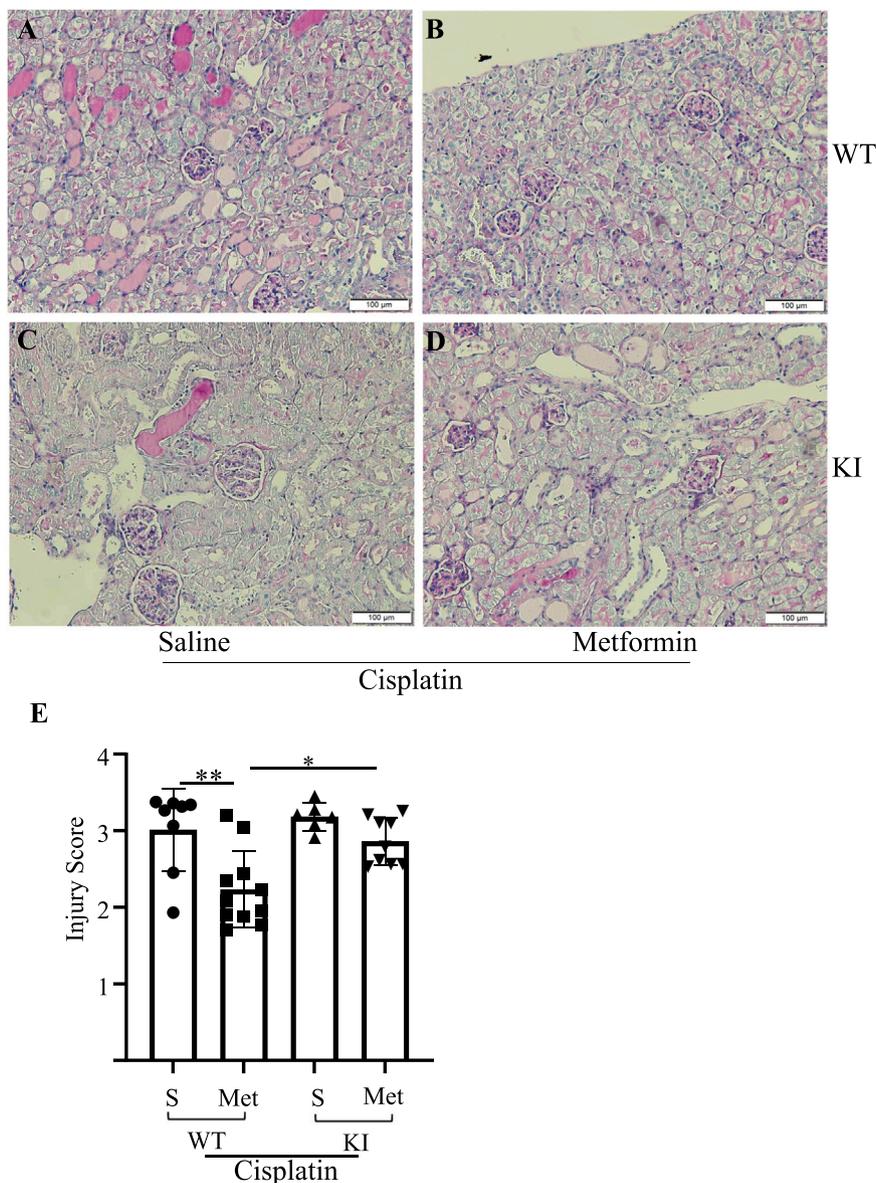


Fig. 7. : The effect of metformin on histological kidney injury, analysed by PAS stain, after CI-AKI in WT and ACC1/2DKI (KI) mice. 7A: WT – saline treated, CI-AKI. 7B: WT – metformin treated, CI-AKI. 7C: ACC1/2DKI – saline treated, CI-AKI. 7D: ACC1/2DKI – metformin treated, CI-AKI. Images were taken at 200x magnification. Scale bar of 100 μ m is shown. 7E: Histological injury score. * $p < 0.02$, ** $p < 0.01$.

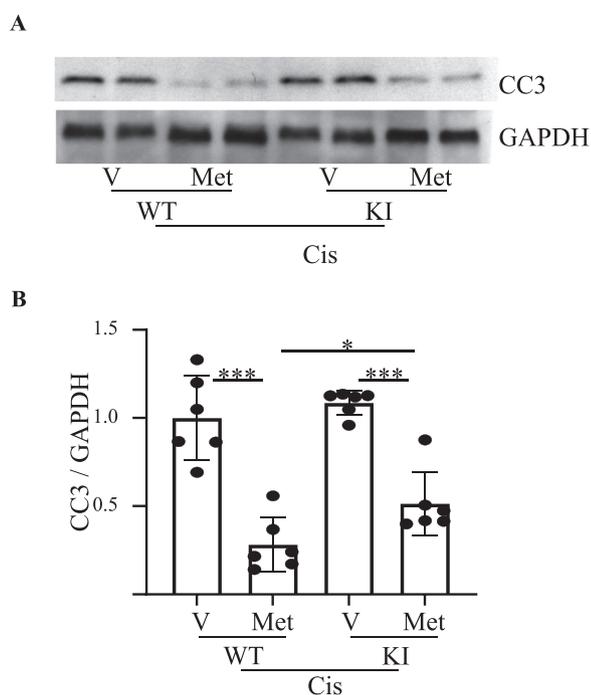


Fig. 8. : The role of ACC phosphorylation in metformin's effect on cisplatin-induced expression of cleaved caspase-3. 8A-B: Primary TECs from female WT and ACC1/2DKI mice were incubated in 4 mM metformin (Met) or vehicle (V) for 4-hours in serum free media, prior to exposure to 20 μ M cisplatin (23 h) (N = 6). Cleaved caspase 3 (CC3) was assessed by Western blot (8 A) for and quantified by densitometry, with GAPDH as a loading control (8B). * $p = 0.03$, *** $p < 0.001$.

metabolites detected were altered (thresholds for difference: fold change (FC) > 1.3, FDR $P < 0.05$) (Fig. 9 A). Notably, the number of altered polar metabolites was markedly increased when comparing metformin-treated ACC1/2DKI and WT cisplatin-AKI kidneys, with a total of 63/217 (29.0%) detected polar metabolites changed (Fig. 9B). In detail, 12 metabolites were found to be higher and 51 were lower in metformin treated ACC1/2DKI CI-AKI mice (Fig. 9B). This difference between the metabolite profiles in WT and ACC1/2DKI mice when treated with metformin, is consistent with the hypothesis that AMPK mediated phosphorylation of ACC contributes to metformin effect on AKI.

In comparing metformin treated, ACC1/2DKI and WT cisplatin-AKI kidneys, heatmap analysis of the 50 most different polar metabolites is shown in Fig. 9 C. With regards to FAs, palmitoleic acid (C16:1) was increased in the ACC1/2DKI samples, whilst the polyunsaturated fatty acids arachidonic acid (C20:4) and eicosapentaenoic acid (C20:5) were decreased, as was cholesterol. No differences in saturated FAs, such as palmitic acid and myristic acid, were observed. Notably, nucleosides such as adenosine, guanosine, uridine, inosine and xanthosine, were reduced in the metformin treated ACC1/2DKI kidneys, as compared to metformin treated WT (Fig. 9B, C). In addition, niacinamide, considered to be protective in AKI²⁶, was also lower in metformin treated ACC1/2DKI kidneys than metformin treated WT (Fig. 9B, C), suggesting that rescue of the kynurenine pathway in CI-AKI by metformin is impaired in ACC1/2DKI mice. Several amino acids, for example L-proline, L-threonine, L-serine, L-tryptophan, L-histidine, and L-isoleucine, were noted to be lower in ACC1/2DKI metformin-treated CI-AKI kidneys (Fig. 9B, C).

Pathway analysis was performed in MetabAnalyst 5.0 comparing polar metabolites from metformin-treated CI-AKI, ACC1/2DKI and WT mice, identifying multiple pathways that are likely to have been altered (Fig. 10). A total of 42 pathways were identified as altered, based on a Benjamini-Hochberg corrected (FDR) likelihood expression threshold of $p < 0.05$, with the pathways with the 20 lowest FDR p-values shown in Fig. 10. Affected pathways included those involving nucleosides, amino

acids, and glucose metabolism.

5. Discussion

FAO is temporarily shut down during AKI [27,28], leading to increased accumulation of lipid in renal tubular cells [29]. Notably, the renal cortex depends primarily on β -oxidation of fatty acids for energy production [5]. Consistent with a previous study [20], in the present study we observed that ACC phosphorylation was reduced in CI-AKI. The present study finds an increased severity of CI-AKI in mice with inability to regulate FA metabolism via phosphorylation of ACC, as evidenced by increased serum urea and creatinine, injury score, and expression of NGAL, IL-1 and IL-6. Furthermore, we found that inability to phosphorylate ACC reduced the protective effect of metformin in CI-AKI, as evidenced by differences in serum urea and creatinine, injury score, cleaved caspase 3, and polar metabolites. Previously, we have described that inability to phosphorylate ACC increases neutral lipid accumulation in the folic acid nephropathy model [8], confirming impaired regulation of FA metabolism in the ACC1/2DKI strain. Furthermore, we also previously reported that inability to regulate ACC phosphorylation increases renal fibrosis with folic acid nephropathy and unilateral ureteric obstruction [8]. The present study suggests that the previous observations of increased renal fibrosis in the ACC1/2DKI strain is likely to reflect, at least in part, a more severe initial kidney injury when regulation of FA metabolism is impaired. ACC is phosphorylated by the energy sensor AMPK [9], and previous studies have found that activation of AMPK is protective against both ischaemic and nephrotoxic forms of AKI [24,30]. The present study indicates that regulation of FAO by ACC phosphorylation is one of the important mechanisms underlying the kidney protective effects of AMPK activation.

In this study, both the ACC1 and ACC2 isoforms were de-phosphorylated. Inhibitory phosphorylation by AMPK at ACC1-Ser⁷⁹ and ACC2-Ser²¹² blocks the conversion of acetyl-CoA to malonyl-CoA, which has two effects [9]. Firstly, FA synthesis is reduced because malonyl-CoA is an intermediate in the synthesis of long-chain FAs. Secondly, FAO increases, due to reduced malonyl-CoA mediated inhibition of CPT1, the enzyme controlling transfer of long-chain fatty acyl CoA into mitochondria [9]. In the present study, CPT1 was markedly reduced at 48-hours with CI-AKI, and this was not different between WT and ACC1/2DKI. This observation may be influenced by the marked severity of kidney injury at 48-hours in this model, which is a limitation that could be addressed in future studies, by examining the effect of CI-AKI on renal CPT-1 expression and other metabolic markers at earlier time points. AMPK activity has been reported to be reduced in CI-AKI in previous studies [20–22]. ACC1 is considered more important in the regulation of FA synthesis, and ACC2 in the regulation of FAO, although there may be some overlap in function [31]. Although the kidney is abundant in mitochondria, especially in proximal tubular cells, our previous studies have found ACC1 to be the dominantly expressed ACC isoform in the kidney [8,32]. Nonetheless, a limitation of the present study is that we have not distinguished the relative contributions of cytoplasmic ACC1 and mitochondrial ACC2 on the observed effects on AKI in the action of metformin. Fullerton et al. found in the liver that single mutations of ACC1 or ACC2 had minimal effect, whereas the combined ACC1/2DKI led to higher hepatic *de novo* lipogenesis [33]. Further future studies will be required to determine the relative contribution of the two ACC isoforms on the outcomes of AKI, as well as on the therapeutic effects of various AMPK activators such as metformin.

The pathogenesis of CI-AKI is complex, involving oxidative stress, endoplasmic reticulum stress, DNA damage, apoptosis, and inflammation [4]. Notably, mitochondrial dysfunction with ATP depletion is recognised as an important contributor to CI-AKI, with findings including reduced mitochondrial density and volume, membrane depolarization, and mitochondrial fragmentation [4,6,10,21]. Consistent with this, in the present study, reduced expression of the mitochondrial

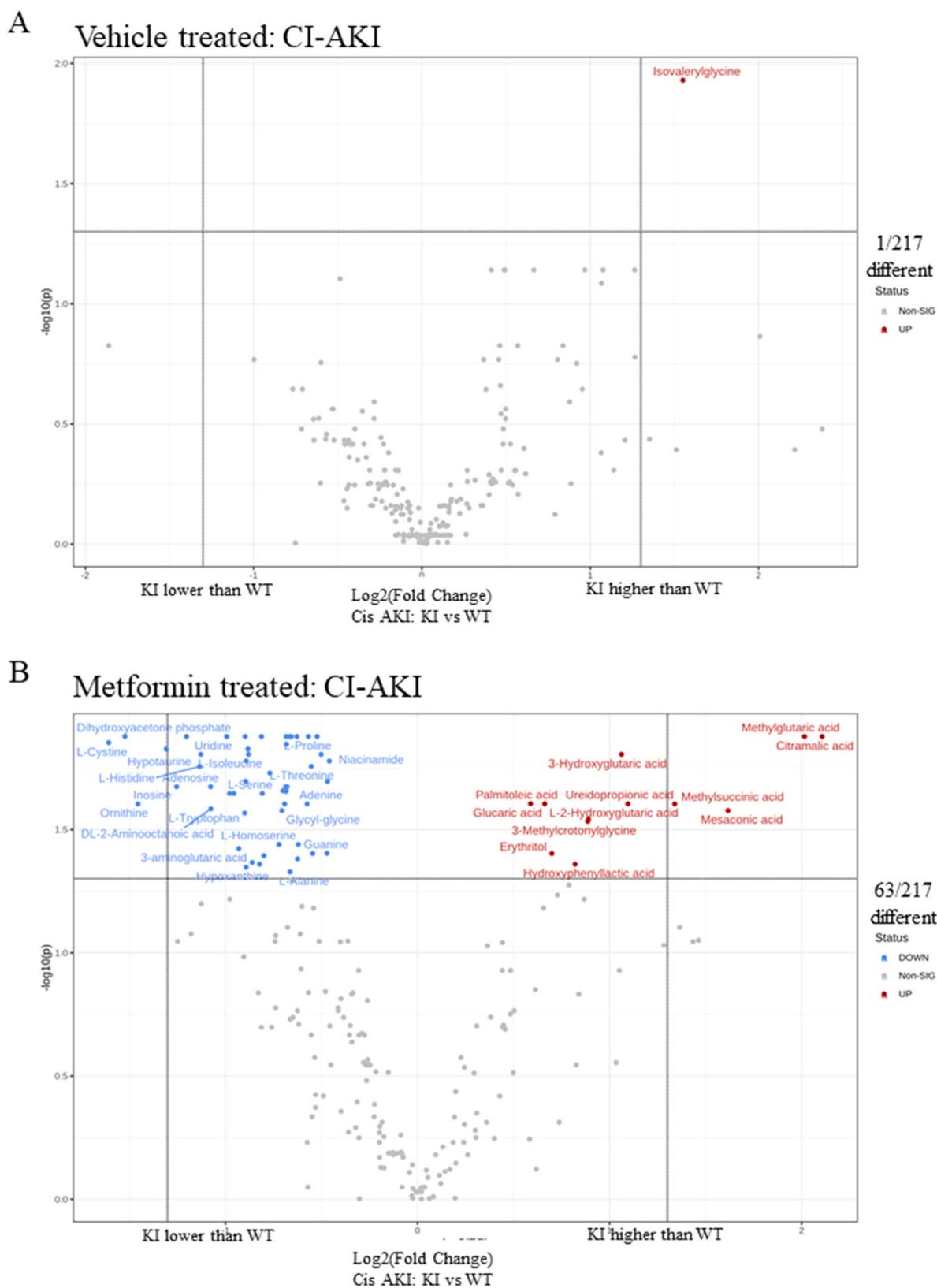


Fig. 9. : Volcano plot analysis of polar metabolites detected by GC-MS in whole kidney CI-AKI samples comparing ACC1/2DKI (KI) and WT treated with vehicle (9 A) or metformin (9B), with x-axis showing log2(fold change), with a 1.3-fold significance threshold, and y-axis showing statistical significance, with a Benjamini-Hochberg corrected (FDR) p-value < 0.05 significance threshold. In the metformin treated analysis 29.0% of metabolites were increased, compared with 0.46% in the vehicle treated analysis. 9 C: Heatmap analysis showing the 50 most different polar metabolites comparing ACC1/2DKI and WT CI-AKI kidney pre-treated with metformin.

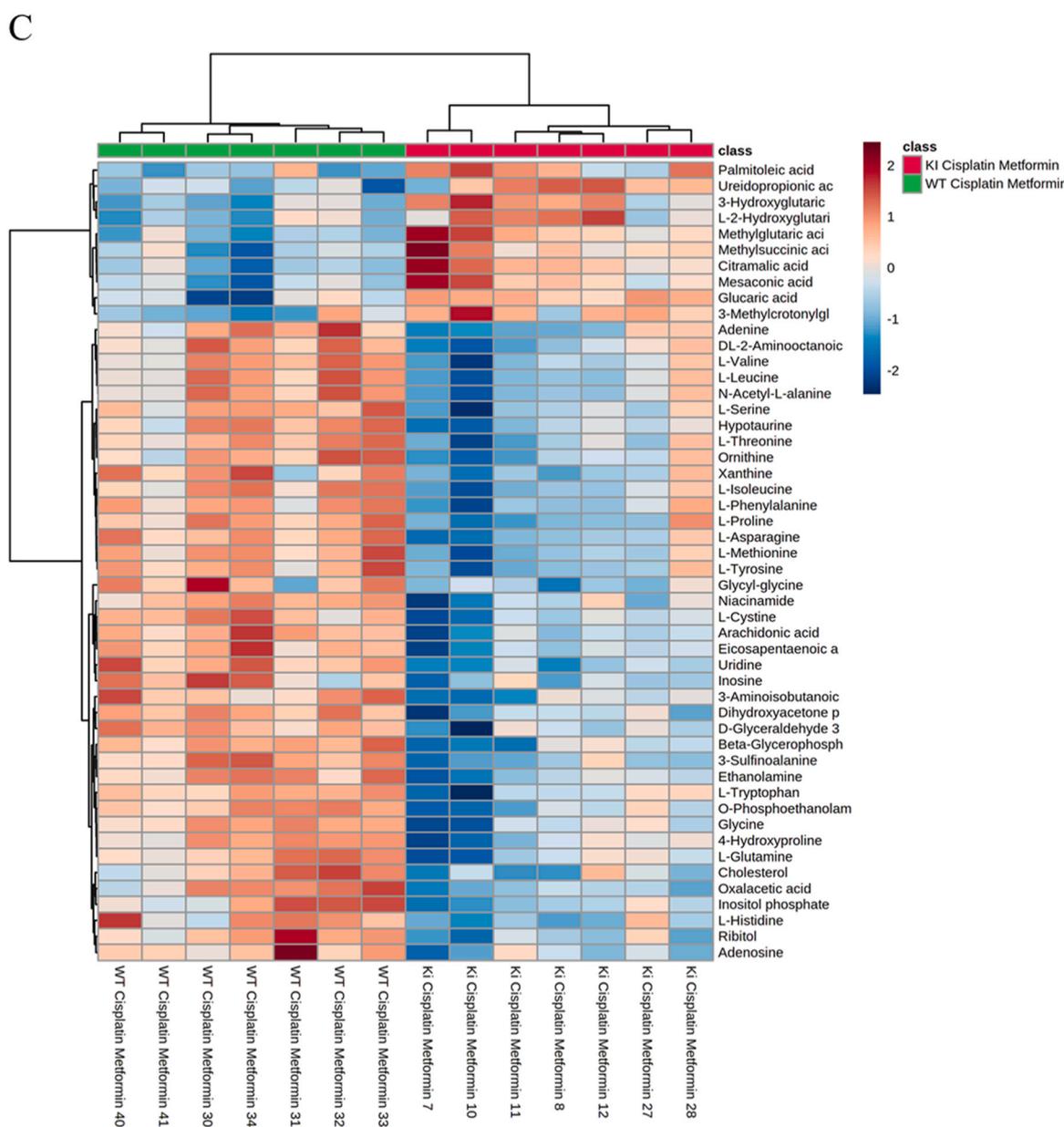
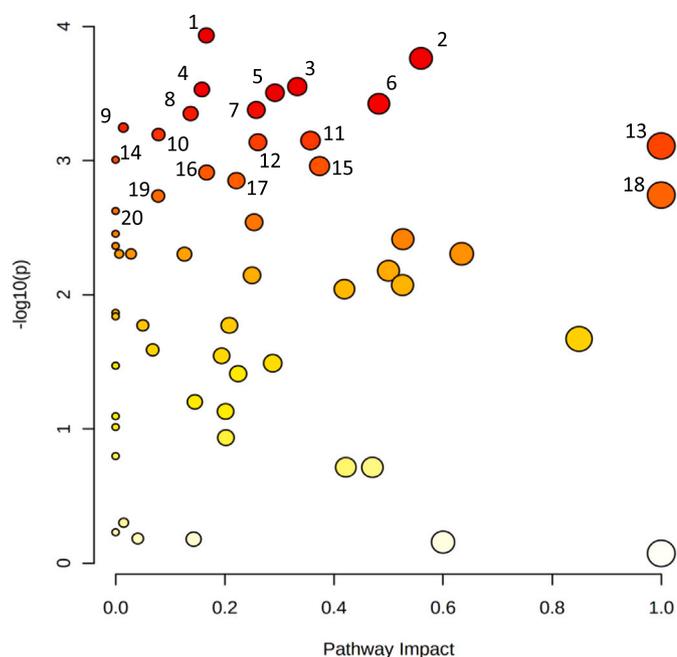


Fig. 9. (continued).

proteins CPT-1 and PCG1 α was observed with CI-AKI. Previous studies have found that metformin confers a protective effect against AKI of various causes including CI-AKI [15,16]. Furthermore, another AMPK activator, AICAR, also protects against CI-AKI, with evidence that improved mitochondrial function contributes to this outcome [21]. In the present study, we observed in the ACC1/2DKI strain, metformin's protective effect was absent in the CI-AKI mouse model and diminished, although not abolished, in the primary cell culture cisplatin cytotoxicity model. Taken together, these data are consistent with there being both ACC dependant and ACC independent contributions to metformin's protective effect in AKI. Previous studies have identified other AMPK-dependant pathways contributing to metformin's protection against kidney injury. These include inhibition of mTOR, induction of autophagy, preservation of sirtuin 3, activation of Akt, and improved mitochondrial function [15,21,30,33]. The present study adds to previous knowledge by demonstrating for the first time the importance of ACC phosphorylation by AMPK, as regulator of FA metabolism, in the nephroprotective action of metformin against toxin mediated AKI.

Analysis of polar metabolites by GC/MS revealed numerous

abnormalities with CI-AKI in both WT and ACC1/2DKI mice, including lower levels of numerous purine and pyrimidine nucleosides, which is similar to findings in a previous study [20]. We also noted disruption of the kynurenine pathway, with evidence in CI-AKI of a block at the level of the enzyme QPRT, responsible for the conversion of quinolinic acid to nicotinic acid [34]. QPRT has been identified as a bottleneck enzyme defending renal NAD⁺ and mediating resistance to AKI [34]. Whilst a previous study has found QPRT deficiency to be a contributor to ischaemic AKI in mice and humans [34], we believe the data in the present study to be the first to extend this observation to a nephrotoxic form of AKI. Previous studies have demonstrated that CI-AKI causes accumulation of lipid and FAs in the kidney, by various methods such as oil-red O staining [20,28,35]. A study by Li et al. demonstrated elevated palmitate in CI-AKI kidney using LC-MS analysis. It is unclear why this increase in palmitate was not also observed in the GC-MS analysis in the present study, although this presumably is related to methodological differences between the studies. An interesting finding in our study was a reduction in polyunsaturated omega-3 FAs with CI-AKI. Of interest, a previous study found that high omega-3 FAs appear to be protective



	Pathway	Impact	FDR P-value
1	Pyrimidine metabolism	0.1669	0.0032
2	Beta-alanine metabolism	0.5597	0.0032
3	Arachidonic acid metabolism	0.3329	0.0032
4	Inositol phosphate metabolism	0.1581	0.0032
5	Glycolysis/gluconeogenesis	0.2921	0.0032
6	Cysteine and methionine metabolism	0.4824	0.0032
7	Fructose and mannose metabolism	0.2577	0.0032
8	Glycerophospholipid metabolism	0.1374	0.0032
9	Sphingolipid metabolism	0.0142	0.0034
10	Pantothenate and CoA biosynthesis	0.0785	0.0034
11	Phenylalanine metabolism	0.3571	0.0034
12	Purine metabolism	0.2608	0.0034
13	Taurine and hypotaurine metabolism	0.9999	0.0034
14	Ubiquinone and other terpenoid-quinone biosynthesis	0.01	0.0040
15	Glycerolipid metabolism	0.3738	0.0041
16	Aminoacyl-tRNA biosynthesis	0.1666	0.0044
17	Histidine metabolism	0.2213	0.0047
18	Phenylalanine, tyrosine and typtophan biosynthesis	1.0	0.0055
19	Primary bile acid synthesis	0.0780	0.0055
20	Valine, leucine and isoleucine biosynthesis	0.01	0.0067

Fig. 10. : Pathway analysis performed in Metaboanalyst5.0 comparing CI-AKI kidneys from metformin-treated ACC1/2DKI vs metformin-treated WT mice. The 20 most statistically different pathways, as determined by Benjamini-Hochberg corrected (FDR) p-value are annotated and listed.

against AKI [36].

In the present study, we observed numerous polar metabolite and pathway differences between metformin treated CI-AKI ACC1/2DKI and WT kidneys. A limitation of the static pool metabolite approach used in the present study is an inability to reliably distinguish between causes and consequences of injury severity. The widespread nature of the differences we observed suggest that these many of these changes may represent downstream consequences of the more severe injury observed in metformin treated CI-AKI ACC1/2DKI kidneys in comparison to metformin-treated CI-AKI WT, reflecting the diminished protection afforded by metformin in the absence of ACC phosphorylation. For example, the lower levels of various nucleosides and amino acids, as well as nicotinamide, may reflect more severe injury, although may also be contributors to more severe injury propagation. Additional future studies will be needed to determine the metabolic patterns of different forms and severities of AKI at various timepoints.

In summary, this study finds that inability to regulate FAO by ACC phosphorylation worsens CI-AKI, supporting the hypothesis that reduced FAO is harmful after AKI. Furthermore, the protective effect of metformin against AKI was impacted by the inability to phosphorylate ACC, demonstrating that regulation of FA metabolism via AMPK-ACC contributes to metformin's nephroprotective effect. These results support the potential for therapeutic approaches directed to kidney FA and energy metabolism as promising strategies to address the therapeutic challenges of acute kidney injury.

CRediT authorship contribution statement

The study was designed by Peter F. Mount. Peter F. Mount and David A. Power acquired the funding. Animal models were performed Geoff Harley, Marina Katerelos and Kurt Gleich. Cell culture experiments were performed by Geoff Harley and Marina Katerelos. Western blot and

histology were performed by Geoff Harley and Marina Katerelos. RT-PCR was performed by Kurt Gleich. Metabolomics were performed by David P. de Souza and Vinod K. Narayana. Figure were prepared by Geoff Harley, Marina Katerelos, Kurt Gleich, David P. de Souza, and Peter F. Mount. Bruce E. Kemp is responsible for the development and supply of the ACC1/2DKI mice. All authors contributed to data analysis. The first draft of the manuscript was written by Peter F. Mount and Geoff Harley. All authors reviewed and edited the manuscript before approving submission of the final version.

Data sharing statement

The data that support the findings of this study are available from the corresponding author, PFM, upon reasonable request.

Conflict of interest statement

The authors have no conflicts of interest to declare in relation to this manuscript.

Data availability

Data will be made available on request.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at [doi:10.1016/j.biopha.2022.113377](https://doi.org/10.1016/j.biopha.2022.113377).

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