



ORIGINAL ARTICLE

The tryptophan pathway and nicotinamide supplementation in ischaemic acute kidney injury

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ABSTRACT

Background. Down-regulation of the enzymes involved in tryptophan-derived nicotinamide (NAM) adenine dinucleotide (NAD⁺) production was identified after acute kidney injury (AKI), leading to the hypothesis that supplementation with NAM may increase the kidney NAD⁺ content, rescuing tryptophan pathways and subsequently improving kidney outcomes.

Methods. Urinary measurement of tryptophan and kynurenin using liquid chromatography–mass spectrometry metabolomics was used in a cohort of 167 cardiac bypass surgery patients along with tests for correlation to the development of postoperative AKI. A mouse model of ischaemic AKI using ischaemia–reperfusion injury (bilateral clamping of renal arteries for 25 min) was also used.

Results. We identified a significant decrease in urinary tryptophan and kynurenin in patients developing AKI, irrespective of the Kidney Disease: Improving Global Outcomes (KDIGO) stage. Although a significant difference was observed, tryptophan and kynurenin moderately discriminated for the development of all AKI KDIGO stages {area under the curve [AUC] 0.82 [95% confidence interval (CI) 0.75–0.88] and 0.75 [0.68–0.83], respectively} and severe KDIGO Stages 2–3 AKI [AUC 0.71 (95% CI 0.6–0.81) and 0.66 (0.55–0.77), respectively]. Sparked by this confirmation in humans, we aimed to confirm the potential preventive effect of NAM supplementation in wild-type male and female C57BL/6 mice subjected to ischaemic

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AKI. NAM supplementation had no effect on renal function (blood urea nitrogen at Day 1, sinistrin–fluorescein isothiocyanate glomerular filtration rate), architecture (periodic acid–Schiff staining) and injury or inflammation (*kidney injury molecule 1* and *IL18* messenger RNA expression). In addition, NAM supplementation did not increase post-AKI NAD⁺ kidney content.

Conclusion. Notwithstanding the potential role of NAM supplementation in the setting of basal NAD⁺ deficiency, our findings in mice and the reanalysis of published data do not confirm that NAM supplementation can actually improve renal outcomes after ischaemic AKI in unselected animals and probably patients.

Keywords: acute tubular necrosis, AKI, biomarkers, cardiac surgery, ischaemia–reperfusion injury

INTRODUCTION

Acute kidney injury (AKI) is a frequent and life-threatening condition. Despite extensive *in vitro* and *in vivo* research, translation of candidate drugs that positively affect renal outcomes in humans has failed so far. In recent years, down-regulation of the enzymes involved in tryptophan-derived nicotinamide (NAM) adenine dinucleotide (NAD⁺) production was identified after AKI, leading to the hypothesis that supplementation with NAM may increase the kidney NAD⁺ content, owing to rescue tryptophan pathways, and subsequently improve kidney outcomes [1].

NAD⁺, a cofactor for redox reactions in the cytosol and mitochondria, is required to maintain the energy supply of cells. NAD⁺ is also a substrate for sirtuins, enzymes that are pivotal for AKI prevention [2–4]. A recent study showed that mice with heterozygous deletion of quinolinate phosphoribosyltransferase (QPRT^{+/-}) develop NAD⁺ deficiency and susceptibility to AKI [5]. Supplementation by NAM increases circulating NAD⁺ in mice and humans, prevents AKI in QPRT^{+/-} mice by rescuing tryptophan-derived NAD⁺ production and may prevent cardiac bypass (CBP) surgery-induced AKI in humans [5]. These results are in line with the hypothesis that reversing disorders of epithelial cell metabolism and mitochondrial dysfunction may improve renal outcomes after ischaemic AKI [6, 7]. Owing to these findings, mainly derived from animal studies, interventional randomized studies in humans are now ongoing in the USA and France to test NAM supplementation after cardiac surgery or renal transplantation (ClinicalTrials.gov NCT04342975 and PHRC 19-0355, respectively). However, conflicting results were recently reported, suggesting that NAM supplementation may not reverse all forms of AKI or CKD [5, 8, 9]. In addition, previous studies showed NAD⁺ deficiency after AKI and that NAD⁺ increased after NAM supplementation in normal mice, but none demonstrated that NAM supplementation actually increases NAD⁺ content after AKI.

In the current work, we aimed to collect further evidence for involvement of the tryptophan pathway in AKI by evaluating the value of urinary tryptophan and/or its derivative, kynurenin, to discriminate between patients who will develop severe AKI following CBP. While we confirm the decrease in urinary tryptophan and kynurenine after CBP in patients developing AKI, albeit with low predictive value, we observed in a mouse model of ischaemic AKI that NAM supplementation does not prevent AKI.

MATERIALS AND METHODS

Patients

From March 2016 to January 2017, we collected urine samples from 509 patients who had a CBP. Patients receiving chronic

renal replacement therapy were excluded from the analysis. Consent of the patients to be included in the clinical and biological collection of the University Hospital of Toulouse was obtained (agreement number DC-2008-463).

Among the initial cohort of 509 patients, we studied the clinical characteristics and urinary concentrations of tryptophan and kynurenin in the 32 patients who developed severe Stages 2–3 AKI ($n=32$), the 70 who developed Stage 1 AKI and the 65 who did not develop AKI (these latter were randomly chosen). Overall, 167 patients were included in this study. The characteristics of the 332 patients with Stage 0 AKI not included in the study can be found in [Supplementary Table S1](#).

Characteristics

Clinical and biological data were collected before, during and after the cardiac surgery. Baseline glomerular filtration rate (GFR) was estimated using the Chronic Kidney Disease Epidemiology Collaboration formula. AKI was staged according to the KDIGO classification established in 2012 ([Supplementary Table S2](#)) [11].

Urinary tryptophan and kynurenin measurements

Urine samples were collected 24 h after the end of the CBP. Urinary concentrations of tryptophan and kynurenin were measured using targeted metabolome analysis (AbsoluteIDQ p180 assay, Biocrates Life Sciences, Innsbruck, Austria). Urinary samples were frozen and shipped to Biocrates Life Sciences. After thawing, samples were centrifuged at 2000 rpm for 5 min at 4°C, extracted and then analysed by liquid chromatography–mass spectrometry (LC-MS). Data were normalized on the urinary creatinine concentration. Missing data (below the limit of detection; $n=3$) were replaced by the mean value of the considered variable divided by 100.

Animal studies

Male ($n=8$) and female ($n=8$) mice (12 weeks old; Janvier Labs, Paris, France) were sedated using isoflurane. Median laparotomy was performed followed by bilateral clamping of the renal arteries for 25 min, according to a previous protocol [5]. NAM or saline vehicle was administered at a dose of 400 mg/kg by intraperitoneal injections. Drugs were given 24 and 1 h prior to the surgery and 4–6 h after the surgery. BUN was measured at Day 2. GFR was measured 1 day before ischaemia–reperfusion and at Day 1, using sinistrin–fluorescein isothiocyanate (FITC) fluorescence measurement [12]. Mice were euthanized at Day 2 following an intraperitoneal pentobarbital injection (180 µg/g). The timing of animal sampling and sacrifice were chosen to reproduce experiments that previously suggested positive effects of NAM supplementation [5]. Animal experiments were approved

Table 1. Characteristics of 167 patients who had cardiac surgery

Parameter	Global (n = 167)	KDIGO 0 (n = 65)	KDIGO 1 (n = 69)	KDIGO 2–3 (n = 33)	P-value 0 versus 1–3	P-value 0–1 versus 2–3
Preoperative characteristics						
Age (years), mean ± SD	69.59 ± 9.7	68.28 ± 10.5	69.6 ± 9.10	72.2.7	0.241	0.090
Male, n (%)	123 (73.7)	51 (78.5)	52 (75.4)	20 (60.6)	0.453	0.155
BMI (kg/m ²), mean ± SD	27.17 ± 4.6	26.96 ± 3.7	27.17 ± 5.1	27.57 ± 5.3	0.629	0.661
Hypertension, n (%)	98 (58.7)	34 (52.3)	43 (62.3)	21 (63.6)	0.333	0.727
Diabetes, n (%)	49 (29.3)	19 (29.2)	19 (27.5)	11 (33.3)	1	0.727
LVEF (%), mean ± SD	56.56 ± 11.2	57.27 ± 12.3	55.65 ± 10.3	57.2 ± 7.11	0.594	0.727
Euroscore II, mean ± SD	3 ± 3	2.1 ± 11.9	3.4 ± 43.6	3.9 ± 0.93	0.005	0.091
Haemoglobin (g/dL), mean ± SD	13.13 ± 1.9	13.73 ± 1.9	13.13 ± 1.9	12.12 ± 1.6	0.008	0.001
Creatininaemia (μmol/L), mean ± SD	108.508 ± 57.9	93.83 ± 20.8	102.102 ± 46.8	150.650 ± 97.7	0.008	0.013
eGFR (CKD-EPI, mL/min/1.73 m ²), mean ± SD	65.15 ± 21.9	70.40 ± 17.5	67.77 ± 21.1	48.98 ± 24.1	0.017	<0.001
eGFR (CKD-EPI), <60 mL/min/1.73 m ² , n (%)	41 (24.6)	20 (30.8)	21 (30.4)	23 (69.7)	0.241	<0.001
Kidney graft recipients, n (%)	5 (3)	0 (0)	2 (2.9)	3 (9.1)	0.241	0.095
Peroperative characteristics						
Surgery, n (%)						
CAB	61 (36.5)	28 (43.1)	22 (31.9)	11 (33.3)		
Valvular	53 (31.7)	19 (29.2)	20 (29)	14 (42.4)		
Combined	31 (18.6)	13 (20)	14 (20.3)	4 (12.1)	0.494	0.527
Thoracic aorta	20 (12)	5 (7.7)	12 (17.4)	3 (9.1)		
Left ventricle	2 (1.2)	0 (0)	1 (1.4)	1 (3)		
Catecholamines, n (%)	153 (91.6)	59 (90.8)	65 (94.2)	29 (87.9)	1	0.625
RBC (yes), n (%)	40 (24)	11 (16.9)	14 (20.3)	15 (45.5)	0.232	0.008
Number of packed RBCs, mean ± SD	0.6 ± 61.1	0.4 ± 40.9	0.5 ± 51.1	1.1 ± 11.3	0.110	0.037
Postoperative characteristics						
Volume expansion (mL/24 h), mean ± SD	1095.809 ± 680.9	961.561 ± 554.2	1155.815 ± 669.9	1234.823 ± 879.3	0.075	0.456
Catecholamines, n (%)	104 (62.3)	34 (52.3)	48 (69.6)	22 (66.7)	0.110	0.727
Sepsis, n (%)	39 (23.4)	7 (10.8)	22 (31.9)	10 (30.3)	0.013	0.570
Iodinated contrast agents, n (%)	15 (9)	4 (6.2)	7 (10.1)	4 (12.1)	0.545	0.625
RBCs (yes), n (%)	70 (41.9)	19 (29.2)	27 (39.1)	24 (72.7)	0.232	0.001
Number of packed RBCs, mean ± SD	1.44 ± 2.6	0.77 ± 1.2	1.22 ± 1.7	3.22 ± 4.5	0.006	0.018
Outcomes						
AKI KDIGO stage, n (%)						
0	65 (38.9)	65 (100)	0 (0)	0 (0)		
1	69 (41.3)	0 (0)	69 (100)	0 (0)	<0.001	<0.001
2	19 (11.4)	0 (0)	0 (0)	19 (57.6)		
3	14 (8.4)	0 (0)	0 (0)	14 (42.4)		
Diuresis 24 h (mL), mean ± SD	1133.813 ± 362.4	1309.530 ± 303.6	1137.413 ± 289.7	78080 ± 356.1	<0.001	<0.001
Death, n (%)	12 (7.2)	0 (0)	2 (2.9)	10 (30.3)	0.013	<0.001

Comparisons were made between patients who developed AKI (KDIGO Stages 1–3) or not (KDIGO Stage 0) and between patients who developed no or mild AKI (KDIGO Stages 0–1) or severe AKI (KDIGO Stages 2–3).

BMI, body mass index (kg/m²); LVEF, left ventricle ejection fraction (%); CKD-EPI, Chronic Kidney Disease Epidemiology Collaboration.

by the local and national ethical committees (CREFRE Inserm/UPS, agreement C31 55507; protocol APAFIS#122-2015-23).

Kidney pathology

Samples of the left kidney were collected and stored in liquid nitrogen or embedded in paraffin. Expression of the Kim-1 and *IL18* genes was assessed by quantitative polymerase chain reaction (StepOnePlus System with SYBR Eurogentec MESABlue, Thermo Fischer Scientific, Waltham, MA, USA) of the messenger RNA (mRNA) extracted using the RNA Easy Minikit (Qiagen, Venlo, The Netherlands). *Hprt* gene expression was used to normalize gene quantification. Four micrometre kidney slices were stained with periodic acid–Schiff and the injury score was

calculated. The kidney injury score was based on the extent of tubular dilatation, epithelial cell flattening or release from the basement membrane and casts within the tubular lumen. Lesions were ranked from 0 to 4 by three blinded observers.

Kidney NAD measurement

NAD kidney content was measured with the NAD/NADH quantification kit (MAK037; Sigma Aldrich, St. Louis, MO, USA). A kidney sample (20–30 mg) was homogenized in NAD⁺/NADH extraction buffer and then centrifuged. Supernatant was diluted (1:2) and the assay was performed according to the manufacturer's instructions.

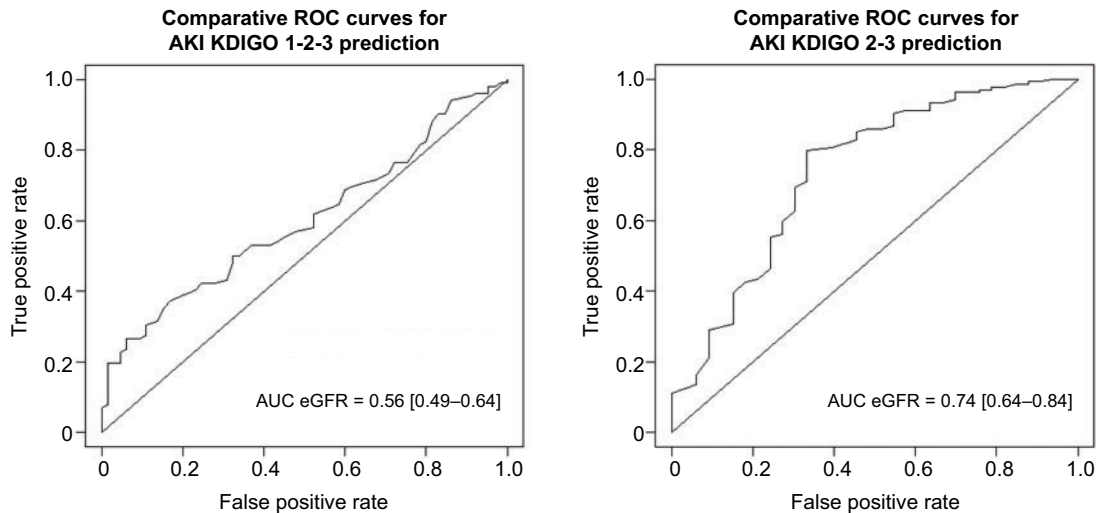


FIGURE 1: Ability of preoperative eGFR to predict the development of (A) AKI (all stages) and (B) severe AKI (KDIGO Stages 2–3) after cardiac surgery. Results are expressed as the AUROC and its 95% confidence interval.

Statistical analyses

Continuous data are represented as mean and standard deviation (SD) and compared with the Wilcoxon test, Aspin–Welch test, Student’s test, analysis of variance (followed by the Tukey post hoc test) or Kruskal–Wallis test (followed by a post hoc pairwise Wilcoxon test), as appropriate. Discontinuous data are represented as number and percentage and compared with the chi-squared or Fischer’s exact test. P-values were adjusted using false discovery correction (Benjamini–Hochberg correction). An adjusted P-value <0.05 was considered significant. The ability of estimated GFR (eGFR) and urinary metabolites to predict the development of AKI and severe AKI was assessed by calculating the area under the receiver operating characteristics curve (AUROC) and 95% confidence interval (DeLong method). Statistical analyses were performed with R version 3.6.1 (additional packages readxl, stringr, ROCR and pROC; R Foundation for Statistical Computing, Vienna, Austria) and Prism 8 software (GraphPad Software, San Diego, CA, USA).

RESULTS

Clinical characteristics

In this study we included 167 patients (mean age 69.5 years) who had CBP. Clinical data are shown in [Table 1](#) and [Supplementary Table S1](#). A total of 102 patients developed AKI, including 33 (19.8%) with severe Kidney Disease: Improving Global Outcomes (KDIGO) Stage 2 or 3 AKI. AKI was diagnosed [maximal serum creatinine (SCr)] at Days 1–6 in 8 (7.8%), 59 (57.8%), 15 (14.7%), 4 (3.9%), 2 (2%) and 14 (13.7%) patients, respectively. Twelve patients died during hospitalization. Mortality was significantly higher in patients who developed AKI [12/102 (11.8%) versus 0/65 (0%); $P=0.004$] and particularly in patients who developed severe Stages 2–3 AKI [10/33 (30.3%) versus 2/134 (1.5%); $P<0.001$].

Univariate analysis showed that baseline estimated glomerular filtration rate (eGFR) and red blood cells (RBCs) transfusion during and after CBP were significantly associated with the risk of developing severe AKI. The AUROC of baseline eGFR (quantitative assessment) was 0.56 [95% confidence interval (CI) 0.49–0.64] for all AKI stages and 0.74 (95% CI 0.64–0.84) for severe AKI prediction ([Figure 1](#)).

Urinary tryptophan after cardiac surgery

In an attempt to confirm at a larger scale the renal changes of the tryptophan pathway in humans developing AKI after CBP, we measured the urinary concentration of tryptophan and kynurenin (a tryptophan derivative) in this cohort of 167 patients using targeted metabolome analysis (AbsoluteIDQ p180 kit, Biocrates Life Sciences, Innsbruck, Austria).

We observed that 24 h post-CBP both urinary tryptophan and kynurenin were significantly reduced in patients developing all AKI stages ([Figure 2A](#)). This was confirmed in patients who developed severe AKI ([Figure 2B](#)). However, the associated AUROC values showed poor discrimination for the development of AKI and severe AKI (AUROCs <0.75) ([Figure 2C and D](#)). Urinary quinolinate could not be assessed because it was not included in the AbsoluteIDQ p180 assay.

Urinary tryptophan and kynurenin were also significantly decreased in patients who died during hospitalization ([Supplementary Figure S1](#)).

NAM supplementation does not prevent ischaemic AKI in mice

Sparked by this confirmation in humans, we studied the potential preventive effect of NAM supplementation in wild-type (wt) male C57BL/6 mice ($n=8$) subjected to ischaemic AKI {bilateral clamping of renal arteries for 25 min [ischaemia–reperfusion injury (IRI)]}. NAM supplementation did not improve sinistrin-FITC clearance-based reduction in GFR 36 h after IRI ([Figure 3A](#)). Blood urea nitrogen (BUN) levels, assessed 48 h after IRI, were also not modified ([Figure 3B](#)). NAM treatment was without effect on renal architecture 2 days after IRI ([Figure 3C](#)). Finally, expression of kidney injury molecule-1 (Kim-1) mRNA, a prominent AKI marker, was even higher in kidneys of NAM-treated mice at Day 2 ($P=0.05$) ([Figure 3D](#)).

Because female gender is associated with less severe AKI following ischaemic AKI, the experiments were reproduced in wt female mice ($n=8$) to address whether NAM can prevent milder forms of AKI. Again, no improvement in AKI with NAM treatment was observed ([Figure 3A–D](#)).

At Day 2, the NAD^+ content within whole kidney (cortex and medulla) of male and female mice that received NAM

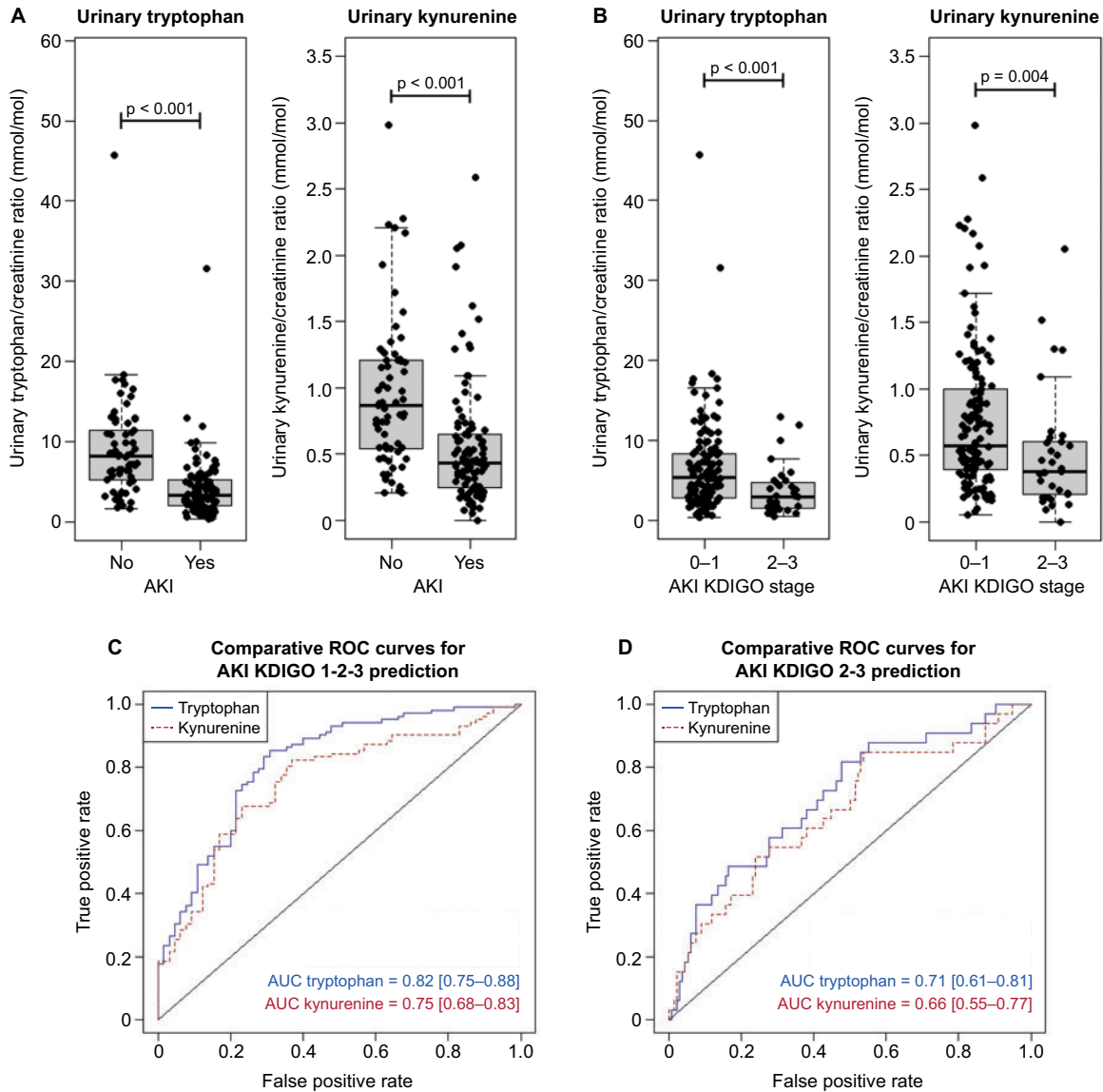


FIGURE 2: Urinary levels of tryptophan and kynurenin 24 h after cardiac surgery according to the AKI stage (KDIGO classification) (A,B). Ability of urinary tryptophan and kynurenin:creatinine ratio to identify patients who will develop all stages of AKI and severe AKI (KDIGO Stages 2–3). Results are expressed as the AUROC and its 95% confidence interval.

supplement was similar to that in mice that received vehicle (Figure 3E).

DISCUSSION

Our findings confirmed, in a large cohort of 167 patients, the initial observation of Poyan Mehr *et al.* [5] in six patients, that urinary tryptophan is reduced in patients who develop CBP-induced AKI, although urinary quinolinate could not be addressed in our cohort because it was not included in the metabolomic assay we used (Biocrates Life Sciences). However, since a strong overlap of urinary tryptophan abundance was observed between groups, tryptophan alone does not appear to be a good marker for the routine detection of early severe AKI.

In contrast to previous studies [5, 6], our results in the IRI mouse model showed that NAM supplementation did not improve AKI outcome. There are a number of differences between our study and these previous studies, despite a similar protocol

for NAM supplementation. The Poyan Mehr *et al.* [5] study used SCr to assess renal function, while we used BUN and measured GFR by inulin-FITC clearance. The use of SCr as surrogate for GFR in mice has been discouraged [10] and it is therefore unclear whether the small differences in the SCr are due to NAM supplementation or other external variables. Hence, depending on the experiments [5, 6], SCr after IRI varied between 0.4 and 1.7 mg/dL and overlapped with SCr in sham mice (0.05–0.7 mg/dL). This overlap does not allow us to adequately assess AKI at the individual level and should prompt, at least, determination of SCr levels before and after IRI at the individual level, or, better yet, to measure GFR before and after experiments, as performed in our study.

The way AKI is assessed in humans in the Poyan Mehr *et al.* [5] study may also be a serious pitfall. Indeed, data suggested that NAM supplementation prevented CBP-induced AKI, but most patients had no AKI or Stage 1 AKI (mean SCr increase was <0.25 mg/dL) and kidney improvement relied on a comparison of three postoperative SCr-derived AUROCs instead of the

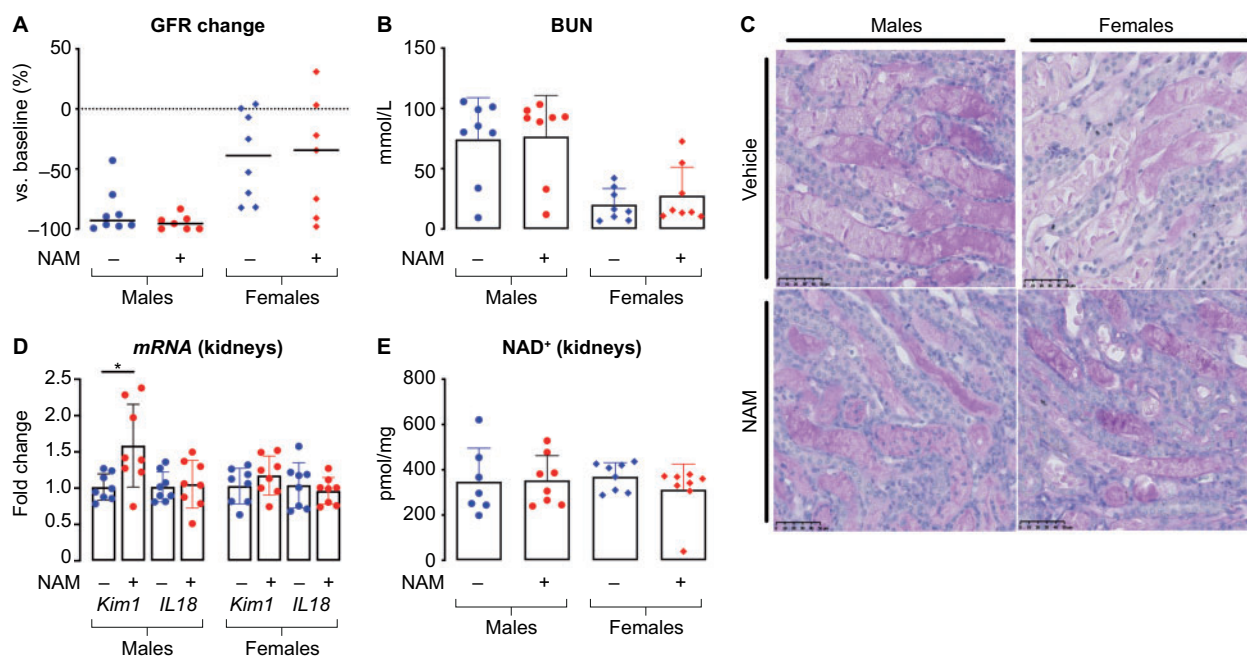


FIGURE 3: Renal outcomes following bilateral kidney IRI after NAM supplementation. (A) Changes of GFR (sinistrin–FITC measurement before and 48 h after bilateral IRI; $n = 8$ per group). (B) Post-ischaemic (24 h) BUN levels in male and female mice ($n = 8$ per group). (C) Kidney pathology of male and female mice after bilateral IRI and vehicle or NAM supplementation. (D) mRNA kidney expression of *Kim-1* and *IL18* genes 48 h after IRI ($P < 0.05$; $n = 8$ per group). (E) NAD^+ kidney content at Day 2 after bilateral kidney IRI.

recommended use of the KDIGO classification. If the latter had been used, the incidence of AKI would not have differed significantly between groups. Moreover, the very small and non-clinically significant difference of SCr between treated and non-treated patients was only observed at Day 1, but NAM supplementation was associated with a decrease of SCr even before the kidney injury (i.e. before the CBP) [5], suggesting interference of NAM in SCr measurements or tubular secretion, challenging the interpretation of SCr curves in humans receiving NAM, and probably in mice.

Furthermore, whereas it was suggested that NAM supplementation can improve renal prognosis after AKI [1, 5, 8], previous data from one of these laboratories showed no improvement of kidney function by NAM supplementation in wt mice submitted to IRI (Figure 1i from references 5, 6), but only effects of NAM supplementation in mice with *Ppargc1a* or *Qprt* deficiency [5, 6]. We clearly confirmed this in our experiments. Whereas we used the same protocol for NAM supplementation, we did not observe improvement of kidney function in male and female wt mice receiving NAM by using measured, not estimated, GFR and BUN. The difference in how kidney NAD^+ deficiency was rescued may also be the origin of these discrepancies: NAM was given via intraperitoneal injection for 1 day before IRI in our study, similar to what is described by Poyan Mehr *et al.* [5], whereas oral administration of NAM riboside was performed by Faivre *et al.* [8]. In the latter study, NAM riboside, a NAM derivative, was started 10 days before bilateral kidney IRI. The employed methodology of the experiments was similar otherwise. Thus further studies may have to better define the optimal regimen to actually rescue NAD^+ deficiency in animal studies and in humans in order to adequately assess efficiency.

Finally, in previous studies the NAD^+ kidney content after NAM supplementation was only assessed at a basal state, but never after AKI. In our experiments, NAD^+ kidney content 2 days after IRI was similar in mice that received or did not

receive NAM supplementation, suggesting that NAM supplementation may actually fail to reverse NAD^+ deficiency in the setting of AKI, at least in wt mice.

CONCLUSION

Notwithstanding the potential role of NAM supplementation in the setting of basal NAD^+ deficiency, our findings in mice and the reanalysis of published data did not confirm that NAM supplementation can actually improve renal outcomes after ischaemic AKI in unselected animals and probably patients. This emphasizes the need to use validated AKI criteria as endpoints in pre-clinical animal models as well as in preliminary studies in humans. This may help to increase the probability of success of interventional placebo-controlled trials in humans.

SUPPLEMENTARY DATA

Supplementary data are available at [ckj](#) online.

FUNDING

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CONFLICT OF INTEREST STATEMENT

None declared.

DATA AVAILABILITY STATEMENT

Data are available upon request to the corresponding author.

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