

# Butyrate promotes kidney resilience through a coordinated kidney protective response in tubular cells

Chiara Favero<sup>a</sup>, Aranzazu Pintor-Chocano<sup>a</sup>, Ana Sanz<sup>a,b</sup>, Alberto Ortiz<sup>a,b,c,\*</sup>, Maria D Sanchez-Niño<sup>a,b,d,\*</sup>

<sup>a</sup> Department of Nephrology and Hypertension, IIS-Fundacion Jimenez Diaz UAM, Madrid, Spain

<sup>b</sup> RICORS2040, Madrid, Spain

<sup>c</sup> Departamento de Medicina, Facultad de Medicina, Universidad Autónoma de Madrid, 28049 Madrid, Spain

<sup>d</sup> Departamento de Farmacología, Facultad de Medicina, Universidad Autónoma de Madrid, 28049 Madrid, Spain

## ARTICLE INFO

### Keywords:

Acute kidney injury  
Microbiota  
Butyrate  
Short-chain fatty acids  
Klotho  
Treatment

## ABSTRACT

Acute kidney injury (AKI) is common in hospitalized patients and increases short-term and long-term mortality. Treatment options for AKI are limited. Gut microbiota products such as the short-chain fatty acid butyrate have anti-inflammatory actions that may protect tissues, including the kidney, from injury. However, the molecular mechanisms of tissue protection by butyrate are poorly understood. Treatment with oral butyrate for two weeks prior to folic acid-induced AKI and during AKI improved kidney function and decreased tubular injury and kidney inflammation while stopping butyrate before AKI was not protective. Continuous butyrate preserved the expression of kidney protective factors such as Klotho, PGC-1 $\alpha$  and Nlrp6 which were otherwise downregulated. In cultured tubular cells, butyrate blunted the maladaptive tubular cell response to a proinflammatory milieu, preserving the expression of kidney protective factors. Kidney protection afforded by this continuous butyrate schedule was confirmed in a second model of nephrotoxic AKI, cisplatin nephrotoxicity, where the expression of kidney protective factors was also preserved. To assess the contribution of preservation of kidney protective factors to kidney resilience, recombinant Klotho was administered to mice with cisplatin-AKI and shown to preserve the expression of PGC-1 $\alpha$  and Nlrp6, decrease kidney inflammation and protect from AKI. In conclusion, butyrate promotes kidney resilience to AKI and decreases inflammation by preventing the downregulation of kidney protective genes such as Klotho. This information may be relevant to optimize antibiotic management during hospitalization.

## 1. Introduction

Acute kidney injury (AKI) is a major public health problem that affects millions of patients worldwide [1]. It is common during hospitalization, developing during admission in around 5 % of patients in general hospitals, especially following surgery [2]. AKI is associated with an increased risk of death persisting for up to a year and of progression to chronic kidney disease (CKD), one of the fastest-growing global causes of death [3,4]. There is no satisfactory treatment that prevents the progression of clinical AKI or accelerates recovery of kidney function, beyond replacing kidney function with dialysis. Most research on the pathogenesis of AKI in search of novel therapeutic targets has focused on cellular and molecular pathways that cause tubular cell

injury such as inflammatory mediators and oxidative stress. Recently, the concept has gained traction that a loss or suboptimal activation of cell and kidney protective pathways may underlie the susceptibility to AKI or to more severe AKI [5–11]. Kidney protective factors that are downregulated in AKI include the antiaging protein of tubular origin Klotho [9], the master regulator of mitochondrial biogenesis PGC-1 $\alpha$  [10,11] and Nlrp6 [8]. Some therapeutic interventions preserve the expression of these kidney protective factors, such as the exogenous administration or overexpression of GDF15 [5] or the preservation of the function of transcription factor Fos11 [6]. However, the eventual clinical implementation of these findings may be decades away given the need to develop and test the efficacy and safety of novel therapeutic agents. The gut microbiota has emerged as a key contributor to homeostasis and an

\* Corresponding authors at: IIS-Fundacion Jimenez Diaz, Av Reyes Católicos 2, 28040, Madrid, Spain; Departamento de Farmacología, Facultad de Medicina, Universidad Autonoma de Madrid.

E-mail addresses: [aortiz@fjd.es](mailto:aortiz@fjd.es) (A. Ortiz), [mdsanchez@fjd.es](mailto:mdsanchez@fjd.es), [mariadolores.sanchez@uam.es](mailto:mariadolores.sanchez@uam.es) (M.D. Sanchez-Niño).

<https://doi.org/10.1016/j.bcp.2024.116203>

Received 10 January 2024; Received in revised form 9 April 2024; Accepted 11 April 2024

Available online 12 April 2024

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altered gut microbiota has been associated with both AKI and CKD [12–16]. It offers a more rapid pathway to clinical implementation through measures already shown to be safe such as optimization of antibiotic management, dietary interventions, prebiotics, probiotics, symbiotics or postbiotics [17]. Among the multiple potential molecules that contribute to the host-microbiota crosstalk, short chain fatty acids (SCFA) derived from dietary fiber such as acetate, butyrate, crotonate and propionate display multiple biological actions that may be beneficial for kidney injury [18,19]. Specifically, butyrate has anti-inflammatory activities [20], was shown to protect from experimental ischemia–reperfusion injury (IRI), gentamicin and contrast-induced AKI or nephropathy [20–24] and is undergoing phase 2 or 3 clinical trials for several conditions [25]. However, the molecular mechanisms of tissue protection by butyrate are not fully understood and the impact on kidney protective molecules has not been addressed.

We have now explored the effect of exogenous butyrate on experimental nephrotoxic AKI induced by either a folic acid overdose or cisplatin and have characterized the molecular mechanisms involved. Specifically, we report that butyrate promotes the kidney resilience to injury by increasing the levels of several nephroprotective factors, including Klotho. Indeed, the parenteral administration of Klotho recapitulated the benefits provided by butyrate.

## 2. Methods

### 2.1. Animal models

Procedures were approved by the animal ethics committee of IIS-FJD (PROEX 036/16). Female (folic acid) or male (cisplatin) 10- to 12-week-old mice were studied. This allowed to test both genders and optimize mice use. Mice were treated with butyrate 100 mM (TCI Chemicals, Toshima, Tokyo) in drinking water for 2 weeks prior to AKI induction and through AKI. Water was changed every week. The oral administration of butyrate mimics the main source of butyrate, which is the processing of dietary fiber by gut bacteria followed by gut absorption. In some experiments, butyrate was stopped prior to AKI induction. Folic acid nephropathy (FA-AKI) is a classical model of AKI [5,6] that has been reported in humans [26]. It was induced by a single intraperitoneal injection of 100 mg/kg folic acid (Sigma-Aldrich, St. Louis, MO, USA) in 0.3 mol/l sodium bicarbonate or vehicle. Cisplatin is a nephrotoxic anticancer agent whose dose-limiting adverse effect is nephrotoxicity. Cisplatin-AKI was induced by a single intraperitoneal injection of 20 mg/kg cisplatin (Sigma-Aldrich, St. Louis, MO) in 0.9 % saline. Control mice received vehicle. The folic acid and cisplatin dose were based on prior experience and dose finding studies. Mice were anesthetized (100 mg/kg ketamine and 15 mg/kg xylazine) at 72 h, which coincides with the peak nephrotoxic effect. Plasma samples were collected (intracardiac puncture), and kidneys were perfused *in situ* with cold saline before removal. One kidney was snap-frozen in liquid nitrogen for RNA and protein studies and the other fixed and paraffin embedded for histological studies [5,6,27].

In a second cisplatin-AKI model, C57BL/6 mice were dosed intraperitoneally with recombinant mouse Klotho (rKlotho; 10 µg/kg, intraperitoneal injection; R&D System, Minneapolis, MN) or vehicle once a day starting one day before the cisplatin injection. The dose of rKlotho was calculated from previous publications [6,28].

### 2.2. Cells and reagents

MCT cells (passage 7) are a cultured line of proximal tubular cells harvested originally from the renal cortex of *SJL* mice and have been extensively characterized [29]. MCT cells were cultured in RPMI 1640, 10 % decomplexed fetal bovine plasma (FBS), 2 mM glutamine, 100 U/ml penicillin and 100 µg/ml streptomycin (all from GIBCO, Grand Island, NY), in 5 % CO<sub>2</sub> at 37 °C. Cells were rested in plasma-free media 24 h prior to the addition of the stimuli and throughout the experiment.

Recombinant human soluble TWEAK (100 ng/ml, Millipore, Burlington, MA), murine TNF-α (30 ng/ml, Peprotech, London, UK) and interferon-γ (INF-γ, 30 U/ml, Peprotech, London, UK) were used for stimulation [5]. Butyrate (1 and 5 mM, Sigma-Aldrich, St. Louis, MO) and the histone deacetylase (HDAC) inhibitor trichostatin A (TSA; 100 ng/ml; Upstate Biotechnology, Millipore, Burlington, MA) were added to cell cultures 1 h before other stimuli [30].

### 2.3. RNA extraction and real-time polymerase chain reaction (PCR)

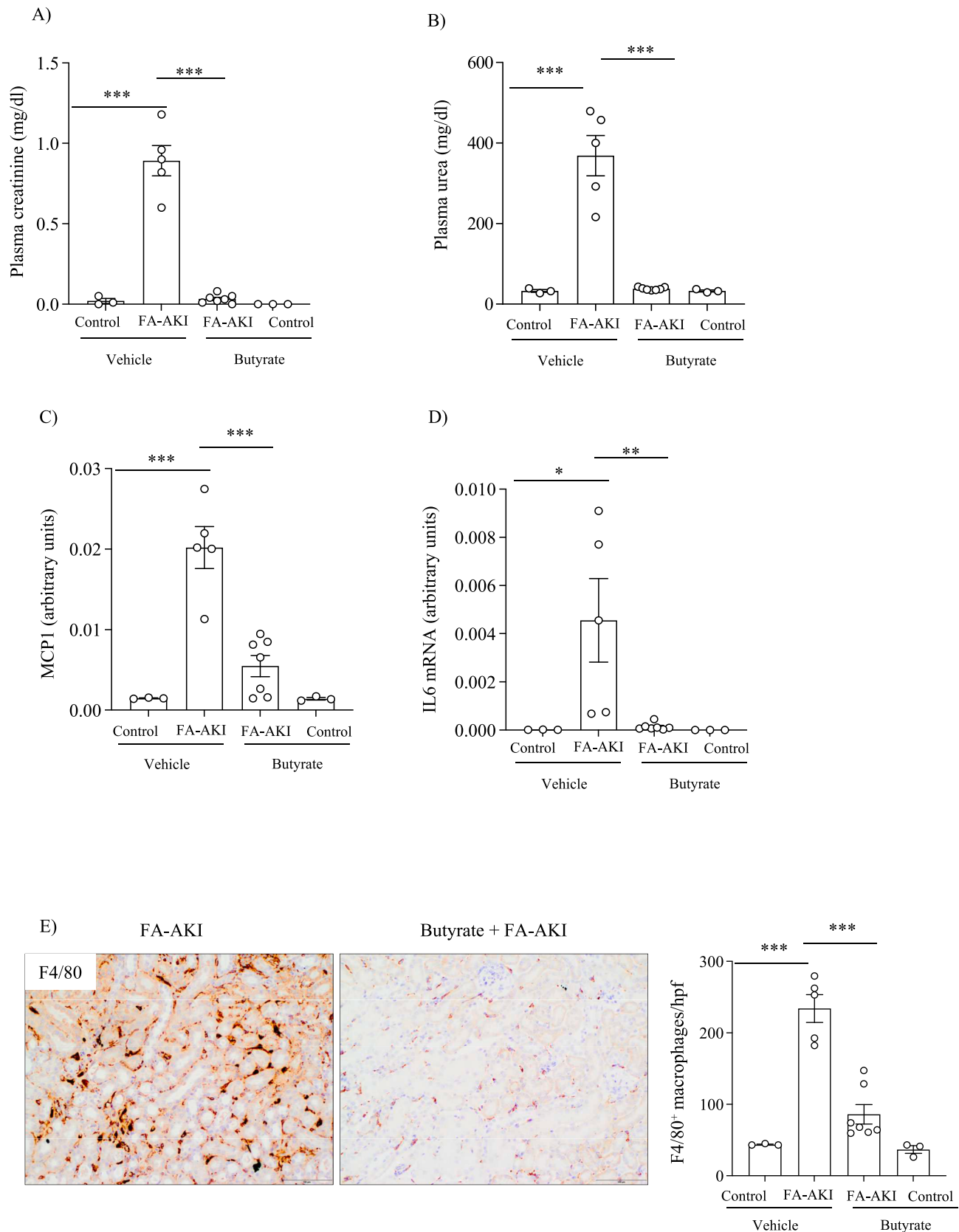
Total RNA was extracted by the TRI Reagent method (Invitrogen, Carlsbad, CA) and 1 µg RNA was reverse transcribed with High-Capacity cDNA Archive Kit (Applied Biosystems, Foster City, CA). Quantitative PCR was performed in a 7500 Real Time PCR System with the Prism 7000 System SDS software using predeveloped primers (Applied Biosystems, Foster City, CA). Expression levels are given as ratios to GAPDH [5,6]. Pre-developed primer and probe assays were from Applied Biosystems, Foster City, CA.

### 2.4. Western blot

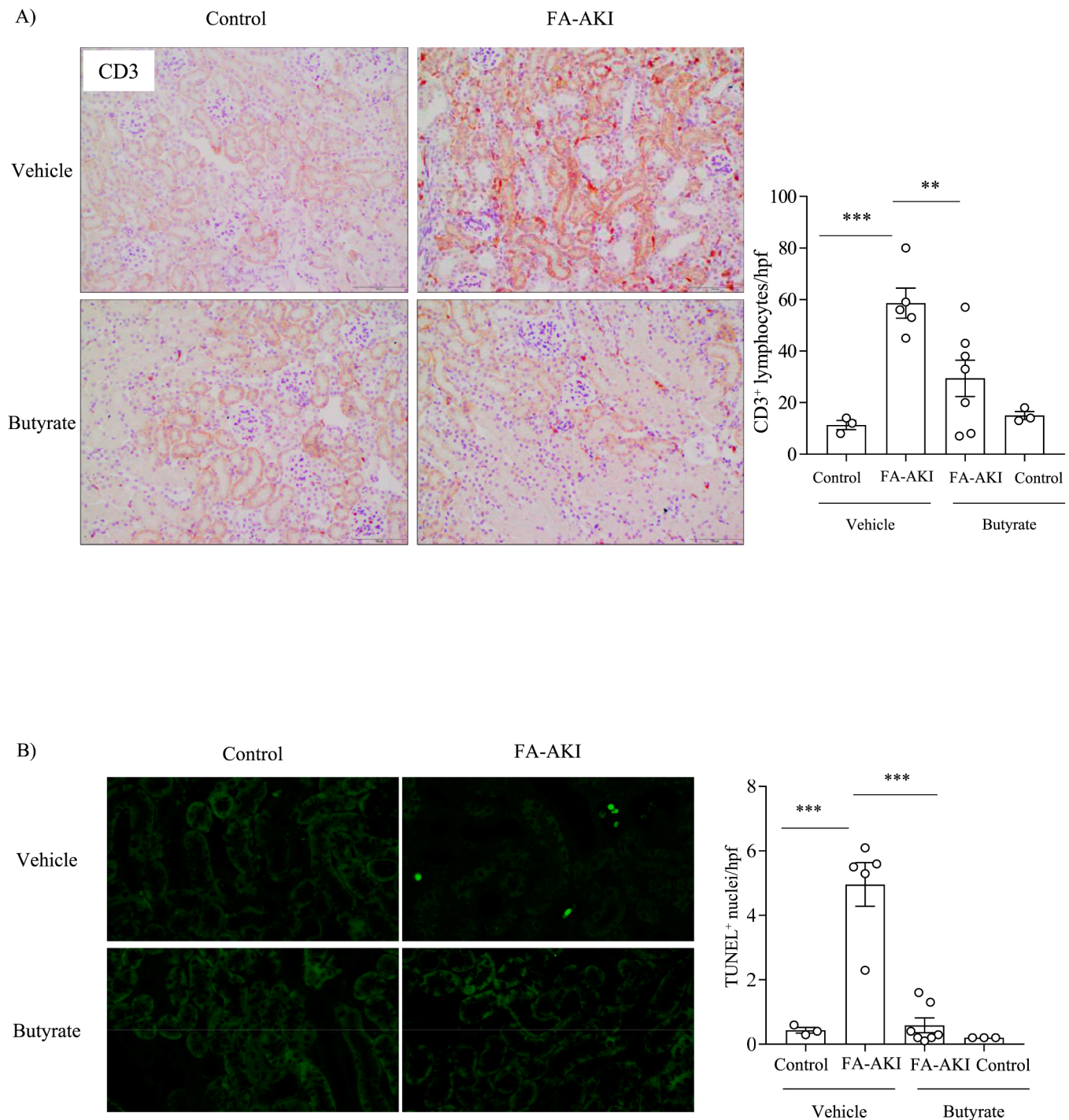
Tissue and cell samples were homogenized in lysis buffer, then separated by 8 %, 10 % or 15 % SDS-PAGE under reducing conditions and transferred to PVDF membranes (Millipore, Burlington, MA, USA), blocked with 5 % skimmed milk in PBS/0.5 % v/v Tween 20 for 1 h, and washed with PBS/Tween [5,6]. Primary antibodies were rabbit polyclonal anti-Klotho (1:1000, Calbiochem, La Jolla, CA), rabbit polyclonal anti-PGC-1α (1:1000, Abcam, Cambridge, UK), mouse monoclonal anti-ERK1/2 (1:1000, Santa Cruz Technology, Dallas, TX), mouse monoclonal anti-pERK (1:500, Santa Cruz Technology, Dallas, TX), rabbit monoclonal anti-nuclear factor kappa B (NFκB) p65 (1:1000, Cell Signaling, Danvers, MA), mouse monoclonal anti-NFκB p50 (1:500, Santa Cruz Technology, Dallas, TX), anti-acetylated H3K9/14 (1:500, Abcam, Cambridge, UK) and rabbit monoclonal anti-phosphorylated p65 (1:1000, Cell Signaling, Danvers, MA). Blots were washed with PBS/Tween, incubated with appropriate horseradish peroxidase-conjugated secondary antibody (1:5000, GE Healthcare, Aylesbury, UK), developed with the chemiluminescence method (ECL) (Fisher Scientific, Waltham, MA) and probed with mouse monoclonal anti-α-tubulin (1:10000, Sigma-Aldrich, St. Louis, MO) and mouse monoclonal anti-Histone 3 (1:1000, Abcam, Cambridge, UK) antibody. Levels of expression were corrected for minor differences in loading.

### 2.5. Histological analyses

Immunohistochemistry was carried out as previously described on paraffin-embedded 3-µm thick sections using the Envision detection kit (Dako) [5,6]. Sections were counterstained with Carazzi's hematoxylin. Primary antibodies were anti-Klotho monoclonal antibody (1:100; Hoelzel Diagnostika, Köln, Germany), rabbit polyclonal anti-PGC-1α (1:50, Abcam, Cambridge, UK), rat polyclonal anti-F4/80 antigen (1:50; Biorad, Hercules, CA), rabbit polyclonal anti-CD3 (1:150, Dako, Glostrup, Denmark), anti-phospho-p44/42 MAPK (Erk1/2) (1:100, Cell Signaling, Danvers, MA) and anti-NFκB p65 (1:50, Cell Signaling, Danvers, MA). Negative controls included incubation with a non-specific immunoglobulin of the same isotype as the primary antibody. TUNEL was performed with the In Situ Cell Death Detection Kit Fluorescein (Roche, Basel, Switzerland) according to the manufacturer's instructions [5]. Representative images were taken using microscopy (Olympus BX53, Tokyo, Japan). Staining was evaluated by a quantitative scoring system, Image-Pro Plus software (Media Cybernetics, Bethesda, MD) in 10 randomly selected fields (x20) per kidney. The total number of CD3 positive cells was quantitated in 10 randomly chosen fields (20x) using also Image-Pro Plus. Samples were examined in a blinded manner.



**Fig. 1.** Butyrate protected from experimental FA-AKI. **A)** Plasma creatinine and **B)** plasma urea. \*\*\* $p < 0.0005$  vs control mice or butyrate-treated AKI mice. **C, D)** Kidney mRNA expression for genes encoding the chemokine MCP-1 (**C**) and the cytokine IL6 (**D**) are lower in butyrate-treated mice with AKI than in WT mice with AKI. qRT-PCR. \* $p < 0.05$ , \*\* $p < 0.005$ , \*\*\* $p < 0.0005$  vs control mice or butyrate-treated AKI mice. **E)** F4/80 macrophage immunohistochemistry showing that macrophage infiltration is lower in butyrate-treated mice with AKI than in WT mice with AKI. \*\*\* $p < 0.0005$  vs control mice or vs butyrate-treated mice with AKI. Data expressed as mean  $\pm$  SEM of  $n = 3-7$  animals per group at 72 h.



**Fig. 2.** Butyrate decreased kidney CD3<sup>+</sup> cell infiltration and cell death in FA-AKI. **A)** CD3 immunohistochemistry and quantification. **B)** TUNEL staining (green) for fragmented DNA characteristic of cell death. The number of CD3<sup>+</sup> T lymphocytes or TUNEL<sup>+</sup> cells was lower in butyrate-treated mice with AKI than in vehicle-treated mice with AKI. Original magnification x20. Data expressed as mean  $\pm$  SEM of  $n = 3-7$  animals per group at 72 h. \*\* $p < 0.005$ , \*\*\* $p < 0.0005$  vs control mice or butyrate-treated AKI mice.

## 2.6. Statistics

Cell experiments were repeated at least three times. Statistical analysis was performed using GraphPad Prism Software 8 (CA, USA). Results are expressed as mean  $\pm$  SEM. Significance at the  $p < 0.05$  level was assessed by Student's  $t$  test for two groups of data and ANOVA for three or more groups with Bonferroni post-hoc correction.

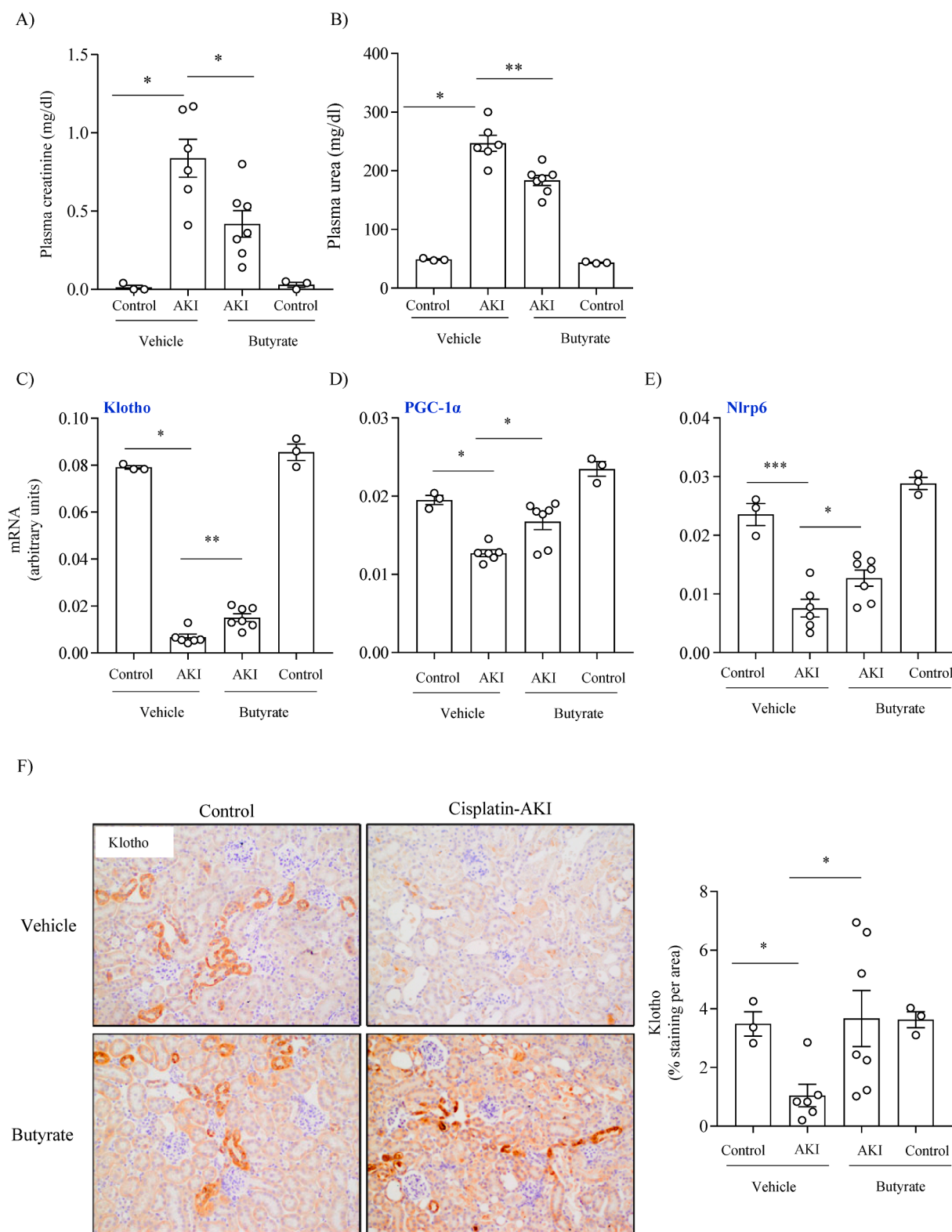
## 3. Results

### 3.1. Butyrate modulates kidney inflammation in AKI

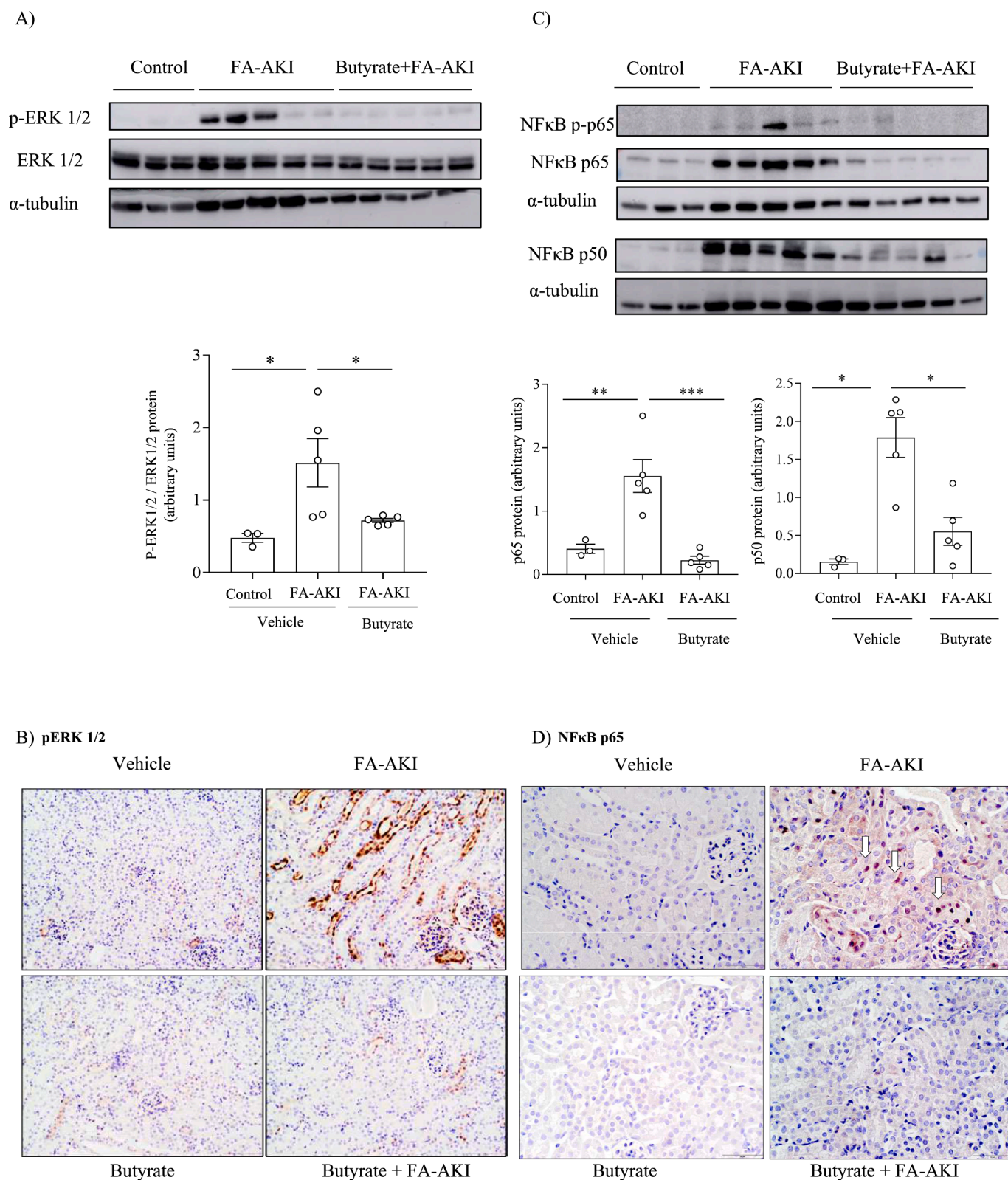
A single folic acid overdose induced AKI in mice, characterized by increased plasma creatinine and urea, kidney expression of chemokines and cytokines and interstitial infiltration by F4/80<sup>+</sup> macrophages and CD3<sup>+</sup> T lymphocytes and cell death at 72 h (Figs. 1-2).

Contrary to findings in a model of a short lived kidney insult, i.e., 30

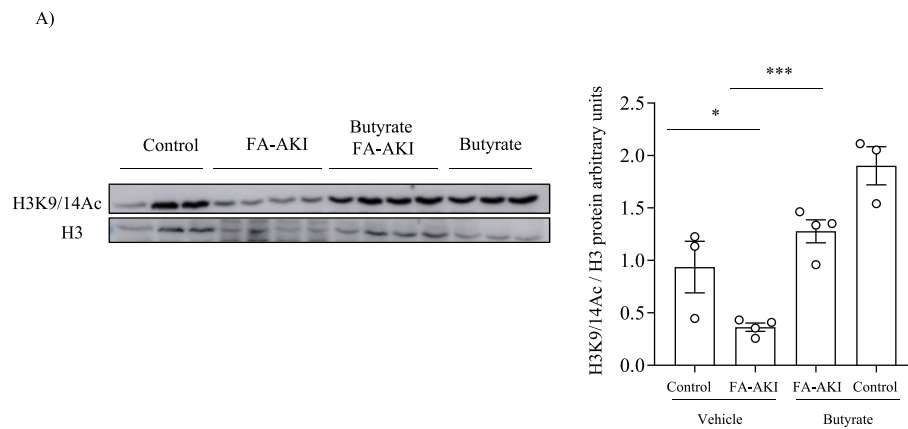




**Fig. 3.** Butyrate protected from experimental cisplatin-AKI. **A)** Plasma creatinine and **B)** plasma urea. **C-E)** Kidney mRNA expression of genes encoding the kidney protective factors Klotho (**C**), PGC-1 $\alpha$  (**D**) and Nlrp6 (**E**) are higher in butyrate-treated mice with cisplatin-AKI than in WT mice with AKI. qRT-PCR. \* $p < 0.05$ , \*\* $p < 0.005$ , \*\*\* $p < 0.0005$  vs control mice or butyrate-treated cisplatin-AKI mice. **F)** Immunohistochemistry showing Klotho expression is preserved in butyrate-treated mice with cisplatin-AKI as compared to vehicle mice with AKI. Original magnification x20. Data expressed as mean  $\pm$  SEM of  $n = 3-7$  animals per group at 72 h.



**Fig. 4.** Butyrate decreases proinflammatory signaling pathway activation in FA-AKI. **A, B)** Butyrate prevented FA-AKI induced kidney ERK phosphorylation. **A)** Western blot, representative image and quantification, **B)** immunohistochemistry localized ERK phosphorylation to tubular cells. **C-D)** Butyrate prevented FA-AKI induced kidney NFκB activation. **C)** NFκB p-p65 and p50 Western blot, representative image and quantification, **D)** immunohistochemistry localized nuclear NFκB p65 to tubular cells. \* $p < 0.05$ , \*\* $p < 0.005$ , \*\*\* $p < 0.0005$  vs control mice or butyrate-treated AKI mice. Data expressed as mean  $\pm$  SEM of  $n = 3-7$  animals per group at 72 h.



**Fig. 5.** Butyrate prevented histone deacetylation in FA-AKI. Representative western blot and quantification. \* $p < 0.05$ , \*\*\* $p < 0.0005$  vs control mice or butyrate-treated AKI mice.

min ischemia–reperfusion injury (IRI) [31], preventive administration of butyrate for two weeks prior to induction of AKI, stopping butyrate prior to AKI, did not decrease the severity of AKI evaluated at 72 h (not shown). Next, we expanded the treatment period by continuing butyrate administration during AKI.

Treatment with oral butyrate for two weeks prior to induction of AKI and during AKI protected from FA-AKI. Plasma creatinine and urea (Fig. 1A,B) and kidney expression of inflammatory mediators (MCP-1 and IL6 mRNA) (Fig. 1C,D) were lower in butyrate-treated mice with FA-AKI than in non-treated mice with FA-AKI. Furthermore, butyrate significantly decreased the kidney infiltration by macrophages (Fig. 1E) and CD3 + T lymphocytes (Fig. 2A). Additionally, butyrate decreased the number of TUNEL + tubular cells representing dying cells (Fig. 2B), which is consistent with the observed preservation of kidney function.

We next tested the impact of oral butyrate in a second model of nephrotoxic AKI, cisplatin-induced AKI. Butyrate also preserved kidney function in mice with cisplatin-AKI as compared to vehicle-treated mice with cisplatin-AKI (Fig. 3A,B).

Overall, butyrate displayed kidney-protective effects in two independent murine models of nephrotoxic AKI.

### 3.2. Butyrate decreased kidney pro-inflammatory signaling in FA-AKI

In the kidney, mitogen-activated protein kinases (MAPKs) such as ERK1/2, transcription factors such as NF $\kappa$ B, and epigenetic changes such as histone deacetylation cooperate to promote inflammation [32–34].

Activation of the ERK1/2 MAPK pathway mediates proinflammatory responses of tubular cells to cytokines [8,35–37]. FA-AKI was characterized by phosphorylation of ERK1/2, indicating ERK1/2 activation in whole kidney extracts (Fig. 4A), which was localized to tubular cells by immunohistochemistry (Fig. 4B). Butyrate decreased ERK1/2 phosphorylation in mice with FA-AKI (Fig. 4A,B).

The NF $\kappa$ B transcription factor is a key regulator of the expression of inflammatory factors in AKI and is activated in tubular cells by various cytokines involved in the pathogenesis of AKI, such as TWEAK [32,38]. AKI increased the kidney protein levels of both components of the canonical NF $\kappa$ B heterodimer, RelA (p65) and NF- $\kappa$ B1 (p50), as well as of phosphorylated p65 and this was prevented by butyrate (Fig. 4C). NF $\kappa$ B activation was also assessed as nuclear translocation of p65 by immunohistochemistry and located to tubular cells. This was also prevented by butyrate (Fig. 4D).

One of the best characterized anti-inflammatory actions of butyrate is inhibition of histone deacetylases (HDAC) [39,40]. In line with this known action, butyrate restored levels of histone acetylation in whole kidneys in mice with FA-AKI in vivo, as compared to the reduced histone acetylation observed in vehicle FA-AKI kidneys (Fig. 5).

These results suggest that butyrate targets multiple signaling

pathways to negatively modulate the inflammatory response in AKI, including downmodulation of ERK1/2 and NF $\kappa$ B signaling and preservation of histone acetylation.

### 3.3. Butyrate preserves nephroprotective factor expression in AKI

In FA-AKI, the kidney expression of the kidney protective genes Klotho, PGC-1 $\alpha$  and Nlrp6 was decreased (Fig. 6A,B, Fig. 7A–C), confirming prior reports [8–10]. Klotho has anti-inflammatory, nephroprotective, and anti-aging properties and preventing the downregulation of Klotho expression protects from AKI [5,6,8,41]. PGC-1 $\alpha$  is the master regulator of mitochondria biogenesis and PGC-1 $\alpha$  deficiency leads to spontaneous kidney inflammation and sensitization to AKI [11]. Nlrp6 has tissue protective properties in gut and kidney [8,42]. The expression of Klotho and PGC-1 $\alpha$  was conserved in butyrate-treated mice with AKI (Fig. 6A–D). Immunohistochemistry localized nephroprotective factor expression to tubular cells and confirmed conserved tubular cell Klotho and PGC-1 $\alpha$  expression in butyrate-treated mice with AKI (Fig. 6D, Fig. 7A,B). Additionally, the kidney gene expression of Nlrp6 was preserved in butyrate-treated mice with FA-AKI (Fig. 7C).

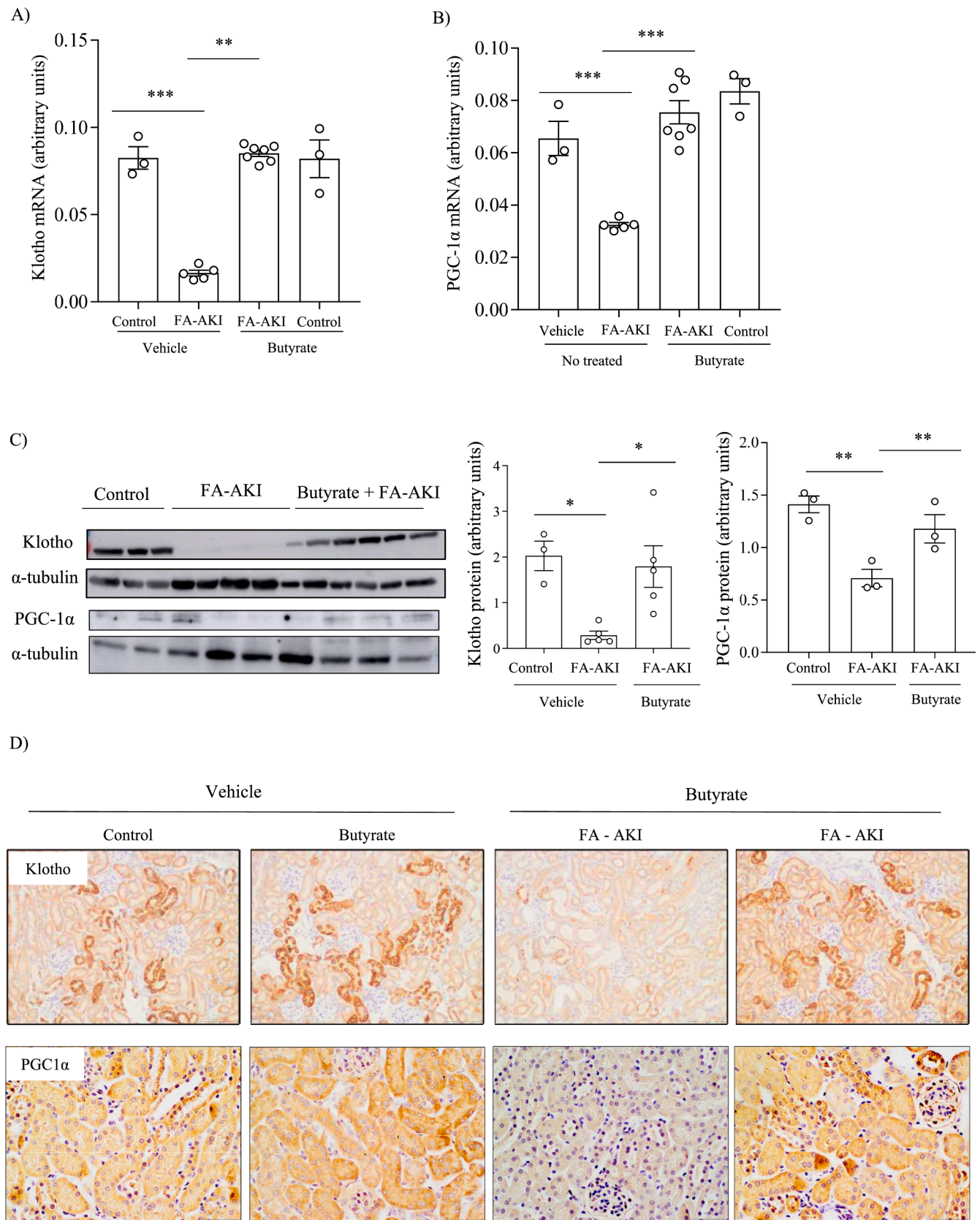
In cisplatin-induced AKI, RT-qPCR confirmed the decrease in nephroprotective factor expression and higher Klotho, PGC-1 $\alpha$  and Nlrp6 expression in butyrate-treated AKI mice (Fig. 3C–E). At the protein level, immunohistochemistry confirmed preservation of Klotho levels in tubular cells (Fig. 3F).

Overall, kidney protection afforded by butyrate was associated with preserved expression of kidney protective factors, consistent with increased kidney resilience, in two independent murine models of nephrotoxic AKI.

### 3.4. Butyrate blunts the maladaptive tubular cell response to a proinflammatory milieu, preserving the expression of kidney protective factors

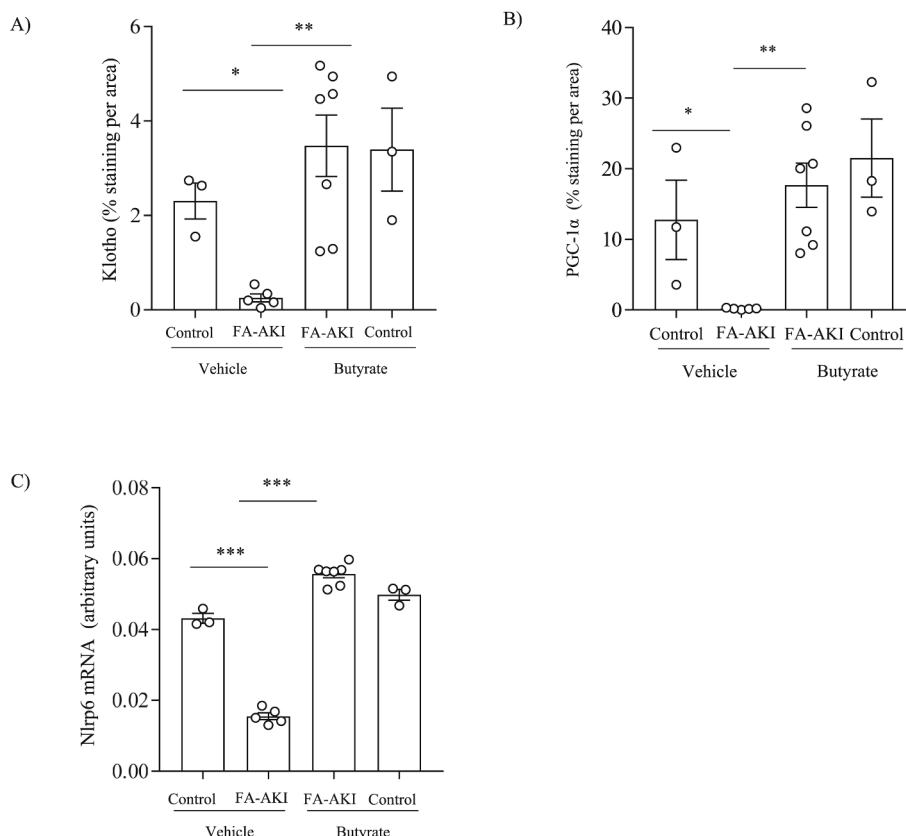
Since immunohistostaining had localized the key events modulated by butyrate to tubular cells in vivo, we next explored in cultured tubular cells the potential mechanisms of nephroprotection afforded by butyrate. Since TWEAK is a key mediator of FA- and cisplatin-AKI, in collaboration with other proinflammatory cytokines such as TNF $\alpha$  and interferon- $\gamma$  [38,43] and in vivo studies had demonstrated that butyrate had to be present during AKI to be protective, we explored the modulation of the response of cultured tubular cells to TWEAK or to a cytokine cocktail containing TWEAK, TNF $\alpha$  and interferon- $\gamma$  in cultured kidney tubular cells. As previously described, TWEAK induced the expression of proinflammatory genes, such as MCP-1 in cultured tubular cells (Fig. 8A) and this response was magnified when TNF $\alpha$  and interferon- $\gamma$





**Fig. 6.** Butyrate preserved nephroprotective factor expression in FA-AKI. **A, B)** Kidney expression for nephroprotective genes Klotho (**A**) and PGC-1α (**B**) was higher in butyrate-treated mice with AKI than in WT mice with AKI. Whole kidney qRT-PCR. **C)** Representative western blot and quantification \* $p < 0.05$ , \*\* $p < 0.005$ , \*\*\* $p < 0.0005$  vs control mice or butyrate-treated AKI mice. **D)** Immunohistochemistry showing Klotho and PGC-1α expression is preserved in butyrate-treated mice with AKI as compared to non-treated mice with AKI and localizes to tubular cells. Original magnification x20 and x40, respectively. Quantification is shown in Fig. 7. Data expressed as mean  $\pm$  SEM of  $n = 3-7$  animals per group at 72 h.





**Fig. 7.** Butyrate preserved nephroprotective factors expression in experimental FA-AKI. **A, B**) Quantification of immunohistochemistry Klotho and PGC-1 $\alpha$  images shown in Fig. 4.D. **C**) Kidney expression of nephroprotective gene Nlrp6 is higher in butyrate-treated mice with AKI than in vehicle mice with AKI. qRT-PCR. Data expressed as mean  $\pm$  SEM of  $n = 3$ –7 animals per group at 72 h. \* $p < 0.05$ , \*\* $p < 0.005$ , \*\*\* $p < 0.0005$  vs control mice or butyrate-treated FA-AKI mice.

were also present in the cell microenvironment (Fig. 9A) [38,43]. Butyrate dose-dependently prevented cytokine-induced proinflammatory responses that are well-characterized to be NF $\kappa$ B-dependent [38], such as the upregulation of the mRNA expression of the representative chemokine MCP-1 (Fig. 8A, Fig. 10A). MCP-1 upregulation was prevented even when stimulated by TWEAK, TNF $\alpha$  and interferon- $\gamma$  (Fig. 9A). Interestingly, the HDAC inhibitor trichostatin A mimicked the effects of butyrate (Fig. 8A).

Proinflammatory cytokines also downregulated the expression of the kidney protective factors Klotho, PGC-1 $\alpha$ , and Nlrp6 in cultured tubular cells (Fig. 8B–D), an effect that was more striking in presence of a more complex proinflammatory environment (TWEAK, TNF $\alpha$ , IFN- $\gamma$ ) that more closely reproduced the complexity of the in vivo environment in AKI (Fig. 9B–E). Again, butyrate dose-dependently prevented the downregulation of kidney protective factors (Fig. 8B–D, Fig. 9B–E, Fig. 10B–D). The positive impact of butyrate was reproduced by adding the HDAC inhibitor trichostatin A, supporting a key role of HDAC inhibition in the observation (Fig. 8B–D). In this regard, butyrate increased histone acetylation in cultured tubular cells, even in the presence of TWEAK (Fig. 11).

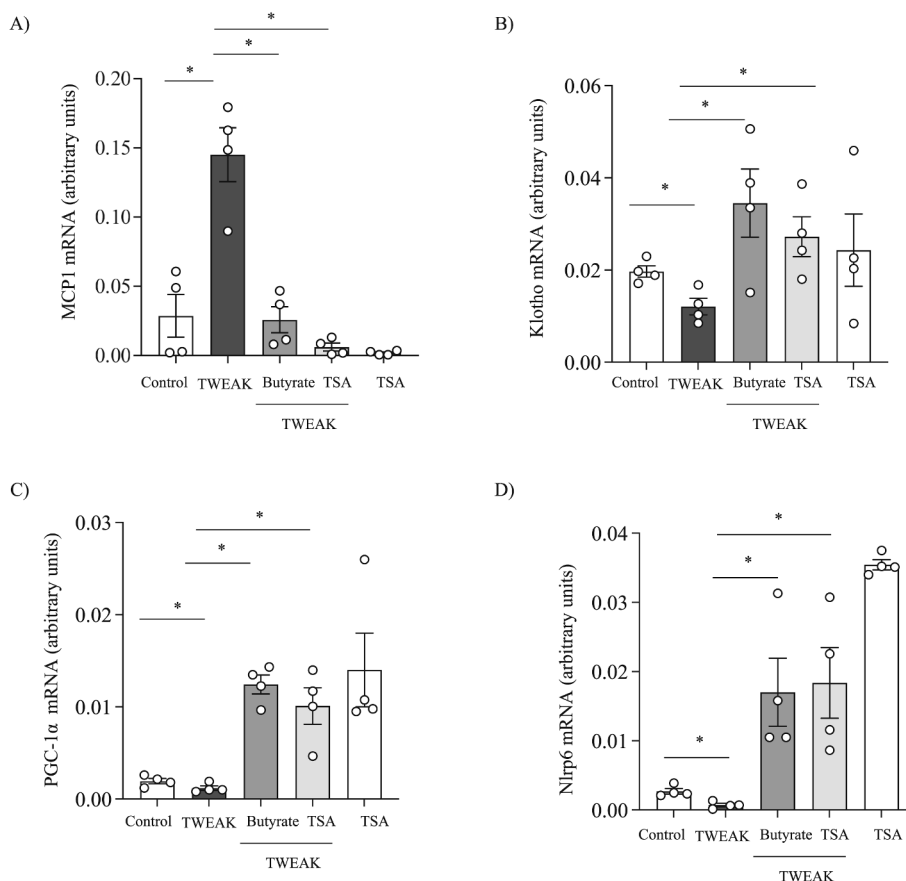
Thus, the anti-inflammatory and nephroprotective effects of butyrate observed in vivo in AKI can be reproduced acutely in cultured tubular cells exposed to inflammatory cytokines and involving inflammatory genes that are upregulated by NF $\kappa$ B [38] as well as nephroprotective genes that are downregulated by NF $\kappa$ B [9,10]. This response is mimicked by the HDAC inhibitor trichostatin A, suggesting that butyrate targets a NF $\kappa$ B-histone acetylation axis that drives both the proinflammatory response and the suppression of tubular cells resilience to stress.

### 3.5. Klotho reproduces the kidney protective effects of butyrate on cisplatin-AKI

Both in cultured tubular cells and in vivo, butyrate decreased inflammation and preserved the expression of kidney protective factors. We next explored whether preservation of kidney protective factors by itself could contribute to the kidney protection offered by butyrate. Of the three kidney protective factors (Klotho, PGC-1 $\alpha$ , Nlrp6), the impact of preserving Klotho can be reproduced in vivo by administering Klotho to mice with AKI. Systemic administration of recombinant Klotho decreased the severity of cisplatin-AKI in mice as assessed by plasma creatinine and urea (Fig. 12A,B) and the kidney expression of MCP1 and IL6 (Fig. 12C,D). Moreover, recombinant Klotho preserved the expression of kidney protective genes PGC-1 $\alpha$  and Nlrp6 in cisplatin-AKI (Fig. 12E,F). Overall, the data support the concept that preservation of Klotho expression, as a representative kidney protective factor, by butyrate has the potential to reproduce the increase kidney resilience afforded by butyrate.

## 4. Discussion

The main finding is the identification of a novel mechanism for the tissue protection offered by butyrate, a representative SCFA of microbial origin, as butyrate coordinately preserved the expression of multiple tissue protective molecules, contributing to the kidney resilience to injury. Although the protective impact of butyrate appeared to be multipronged, administration of recombinant Klotho to mice with AKI, to recapitulate the preservation of Klotho expression by butyrate, was able to reproduce the key features of butyrate kidney protection. This illustrates the contribution to kidney resilience of preserving the expression of kidney protective molecules, exemplified by Klotho.



**Fig. 8.** Butyrate and the HDAC inhibitor trichostatin A (TSA) prevented TWEAK-induced upregulation of MCP1 and downregulation of kidney protective factors in cultured tubular cells. Cell extract mRNA expression for MCP-1 (A), Klotho (B), PGC-1 $\alpha$  (C) and Nlrp6 (D). qRT-PCR. \*p < 0.05 vs control or vehicle TWEAK.

Additionally, the need to continue administering butyrate as AKI developed upon exposure to a nephrotoxin was established. This information may help to optimize clinical care and design clinical trials. In this regard, the widespread use of antibiotics in hospitalized patients will decrease gut bacteria, leading to deprivation of a key source of butyrate at a time with continuous exposure to butyrate is key for the resilience of the kidneys (and potentially other organs).

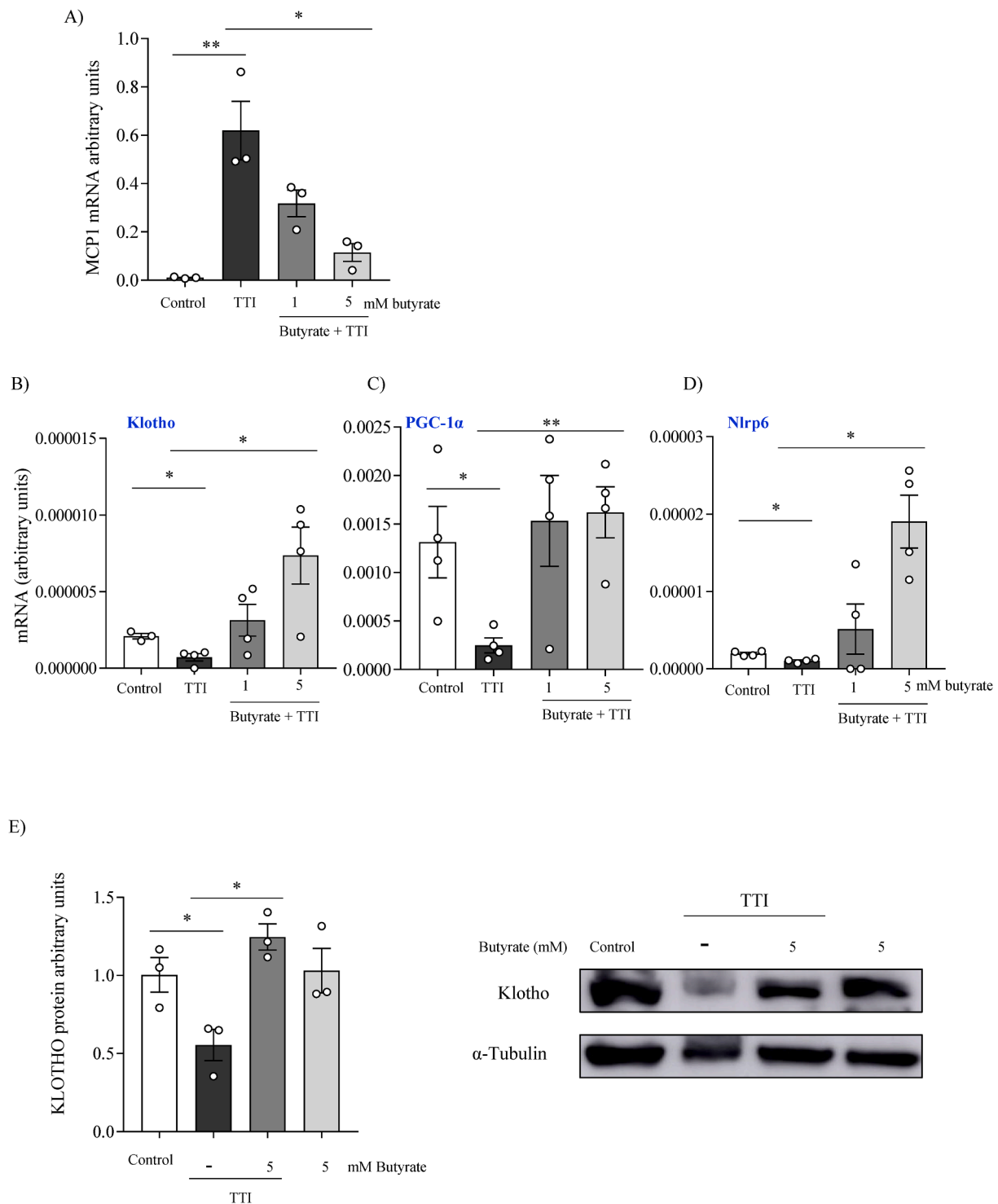
The role of the gut microbiome in AKI was demonstrated by the more severe tubular injury in germ-free mice after kidney IRI than control mice, and decrease in AKI severity by conventionalizing the germ-free mice with normal bacteria [44]. SCFAs, including acetate, propionate, and butyrate, are produced by the gut microbiota from dietary fiber, possess anti-inflammatory actions, and are thought to be key molecules in tissue protection offered by the gut microbiota [45]. SCFA may activate specific receptors (e.g., GPR41, GPR43, Olfir78, GPR109a). Butyrate also inhibits HDAC resulting in the relative hyperacetylation of core histone proteins (H3 and H4), which also confers anti-inflammatory properties [46]. In addition, SCFA suppresses NF $\kappa$ B activation, which is a well-known proinflammatory transcription factor [47]. Our results are aligned with these general features of the anti-inflammatory impact of SCFA, but they also identify a novel mechanism of action, which consists of increasing kidney resilience to injury by preserving the expression of kidney protective factors that are suppressed by NF $\kappa$ B and histone deacetylation in response to tubular cell stress [9,10].

Only two prior studies had demonstrated a kidney protective effect of oral butyrate in experimental AKI [31,48]. However, they differed in the schedule of butyrate administration. Oral butyrate starting two weeks prior to ischemia protected from IRI-AKI [31] while oral butyrate was continued during AKI in studies that showed protection from FA-AKI [48]. Thus, it remained unclear whether butyrate administration

during AKI was required for protection from nephrotoxic AKI. We have now shown that continued butyrate administration is required for protection from FA-AKI. As for other forms of nephrotoxic AKI, FA-AKI slowly develops over time: a first wave of ferroptotic cell death already decreases kidney function and triggers an inflammatory response that amplifies kidney injury leading to peak plasma creatinine at 72 h [49]. In cisplatin nephrotoxicity, plasma creatinine also increases over 72 h [5]. In this regard, cell culture studies support an ongoing need of butyrate presence to be protective, as it had an acute impact on epigenetic events (inhibition of histone deacetylation) in cultured tubular cells acutely stressed by exposure to inflammatory cytokines. A *bona fide* HDAC inhibitor, trichostatin A, reproduced butyrate actions in this cell system.

Both prior studies of oral butyrate focused on the impact of butyrate on kidney function and on molecular pathways that cause kidney injury, mainly inflammation and oxidative stress, but they did not address the impact of butyrate on kidney resilience and kidney protective molecules. Additionally, parenteral butyrate was also protective in rat or murine AKI induced by contrast medium, gentamicin and ischemia–reperfusion injury [20–24]. Again, studies on parenteral butyrate only focused on kidney function, kidney injury and mediators of kidney injury, without exploring kidney protective factors.

Our study, in addition to confirming prior reports of the anti-inflammatory effect of butyrate on diverse cell types through inhibition of NF $\kappa$ B activation and histone deacetylation [46,47], also characterized the preservation by butyrate of the tubular cell expression of a set of molecules that have kidney protective properties (Klotho, PGC-1 $\alpha$ , and Nlrp6) and are coordinately downregulated during tubular cell injury, in cultured cells or in vivo, through the combined action of histone deacetylation and NF $\kappa$ B activation upon exposure to insults that

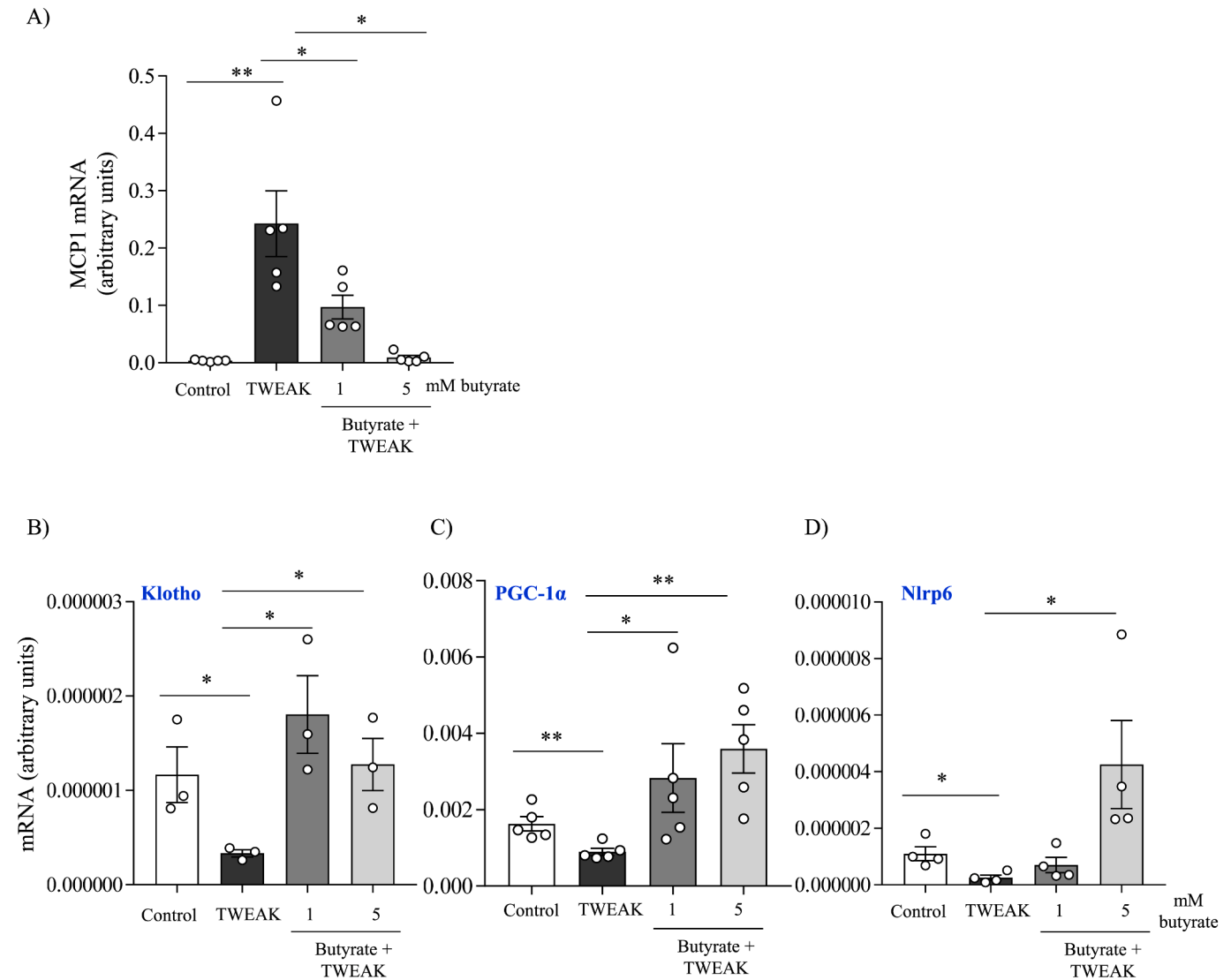


**Fig. 9.** Butyrate prevented TWEAK, TNF- $\alpha$  and interferon- $\gamma$  (TTI)-induced upregulation of MCP1 and downregulation of kidney protective factors in cultured tubular cells. Tubular cell mRNA expression for MCP-1 (A), Klotho (B), PGC-1 $\alpha$  (C) and Nlrp6 (D). qRT-PCR. E) Klotho representative western blot and quantification. \* $p < 0.05$ , \*\* $p < 0.005$ , vs control or butyrate 5 mM- TTI.

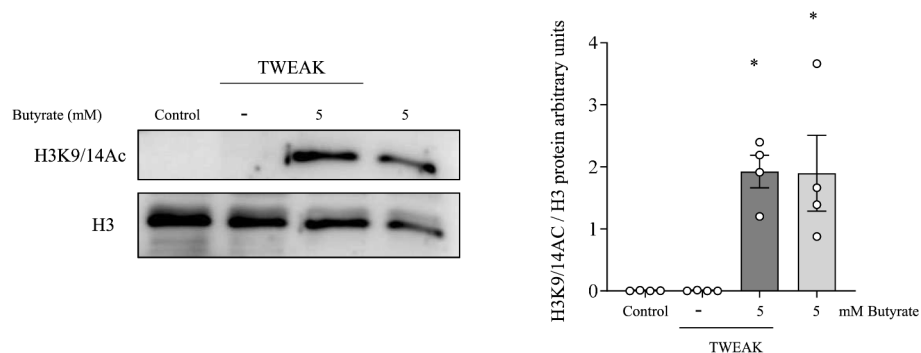
range from inflammatory cytokines to albumin [9,10,30]. Among these kidney protective molecules preserved by butyrate, we chose Klotho for proof-of-concept studies that demonstrated that preservation of one of the kidney protective molecules played a key role in nephroprotection offered by butyrate, as it reproduced features of butyrate protection from cisplatin-AKI. Klotho was chosen because it can be administered parenterally, as it is an extracellular protein. By contrast, PGC-1 $\alpha$  and Nlrp6 are intracellular molecules, and selectively preserving their

expression would require genetic interventions that may not exclusively preserve their expression in tubular cells.

The findings could have a direct translational impact both through a better understanding of the potential role of antibiotics in hospital-acquired AKI and on the design of clinical trials exploring therapeutic strategies with butyrate. While some antibiotics (e.g., aminoglycosides, vancomycin) are known to be nephrotoxic, additional non-nephrotoxic antibiotics have been identified as independent risk factors for in-



**Fig. 10.** Butyrate prevented TWEAK-induced upregulation of MCP1 and downregulation of kidney protective factors in cultured tubular cells: dose-dependency. Tubular cell mRNA expression for MCP-1 (A), Klotho (B), PGC-1α (C) and Nlrp6 (D). qRT-PCR. \*p < 0.05, \*\*p < 0.005, vs control or butyrate- TWEAK.

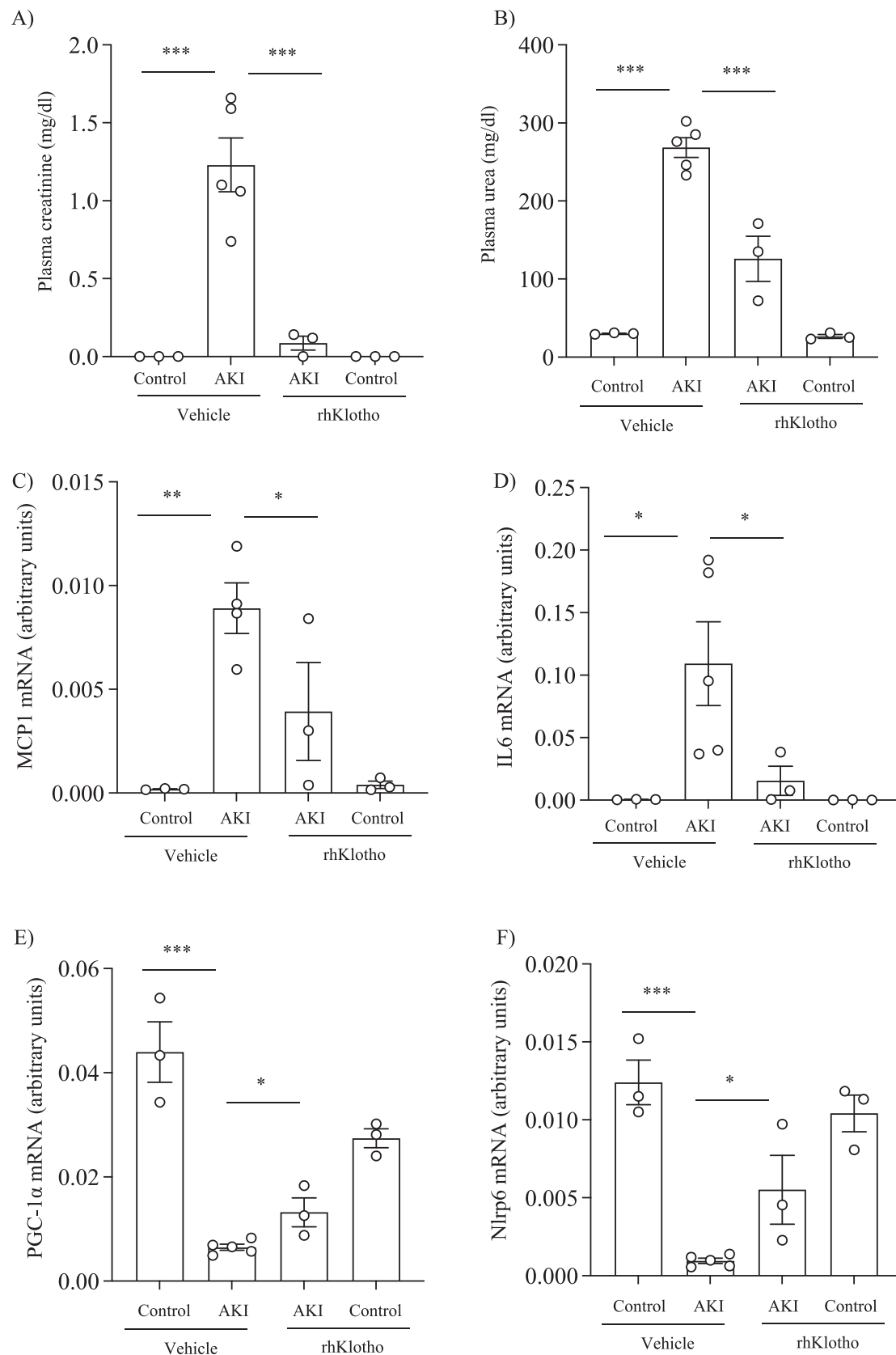


**Fig. 11.** Butyrate increased histone acetylation in TWEAK-stimulated cells. Representative western blot and quantification. \*p < 0.05 vs TWEAK alone.

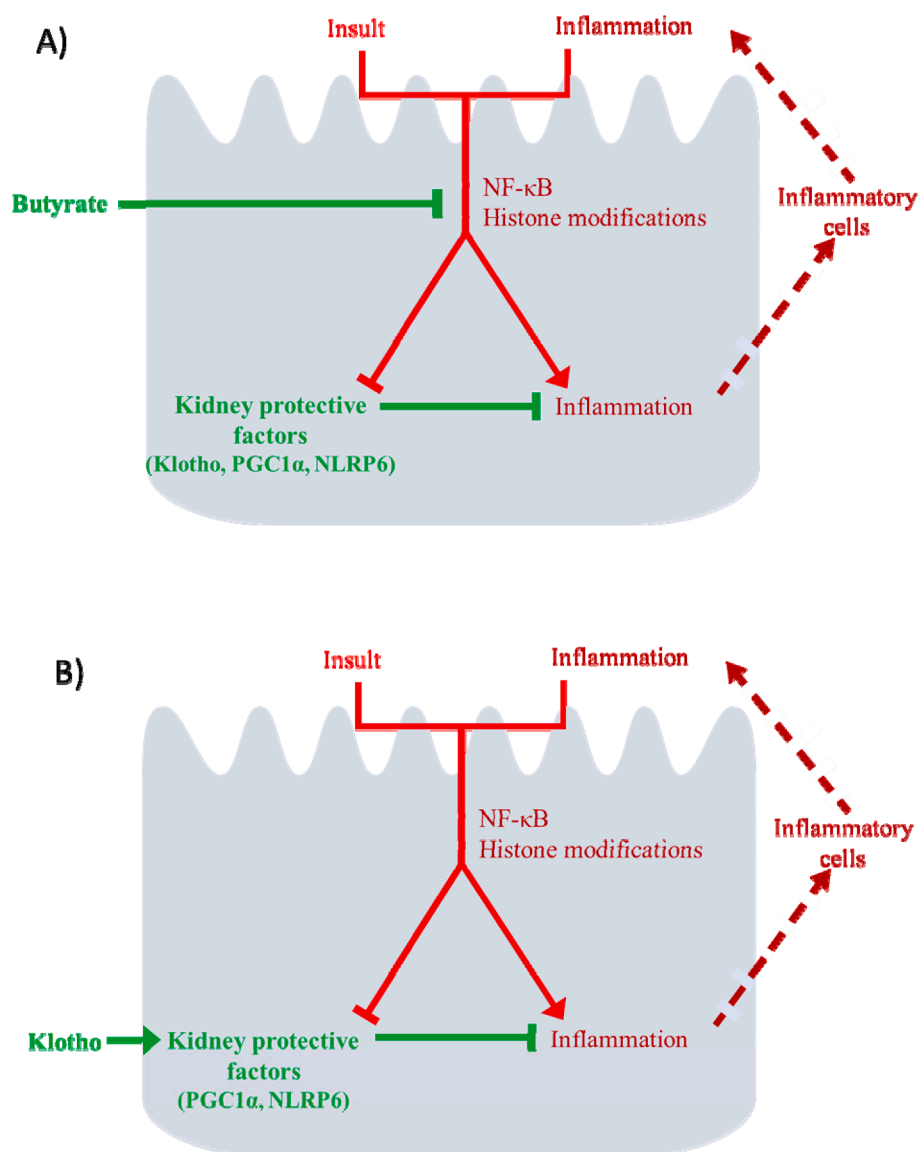
hospital AKI, as recently observed for piperacillin-tazobactam [50]. These wide-spectrum antibiotics would be expected to have a negative impact of the gut microbiota, potentially decreasing butyrate availability during hospitalization, independently of butyrate availability prior to hospitalization, somehow reproducing the experimental setup in which prior exposure to butyrate did not prevent FA-AKI. Interestingly,

the association of piperacillin-tazobactam with AKI was recognized very recently, especially when associated with another wide-spectrum antibiotic with nephrotoxic potential (vancomycin), i.e., under conditions expected to further compromise butyrate availability just when confronting a nephrotoxic insult [51]. On top of providing the rationale to optimize antibiotic prescription in a kidney-friendly manner, these data





**Fig. 12.** The systemic administration of recombinant Klotho decreases the severity of AKI and kidney inflammation and preserves the expression of other kidney protective genes at 72 h. **A)** Plasma creatinine. **B)** Plasma urea. **C-F)** Kidney mRNA expression for MCP-1 (**C**), IL6 (**D**), PGC-1α (**E**) and Nlrp6 (**F**). qRT-PCR. \* $p < 0.05$ , \*\* $p < 0.005$ , \*\*\* $p < 0.0005$  vs control mice or AKI mice. Data expressed as mean  $\pm$  SEM of  $n = 3-5$  animals per group at 72 h.



**Fig. 13.** Conceptual representation of the impact of butyrate increasing kidney resilience by preserving kidney protective genes. **A)** Either the initial insult or inflammation promotes the activation of NFκB and histone deacetylation in tubular cells. As a result, genes encoding inflammatory mediators are transcribed, potentially amplifying inflammation and injury, while genes encoding kidney protective genes are suppressed. Through inhibition of NFκB activation and histone deacetylation, butyrate decreases inflammation and preserves the expression of kidney protective genes. **B)** To assess the relative contribution of the preservation of kidney protective genes, mice were administered recombinant Klotho, which reproduced the effect of butyrate increasing kidney resilience by preserving the expression of other kidney protective genes and decreasing kidney inflammation.

may serve to design therapeutic interventions using butyrate in high-risk populations such as those treated with piperacillin-tazobactam-vancomycin. In this regard, only some clinical trials of oral butyrate in conditions such as inflammatory bowel disease, obesity and diabetes have been promising, illustrating the need to better understand the molecular mechanisms of tissue protection [52–57]. Currently, five ongoing phase 2 trials and one phase 3 trial, totaling 285 participants, are exploring the impact of oral butyrate in conditions such as rheumatoid arthritis, ulcerative colitis, graft versus host disease, Gulf War illness, diabetes and Hirschsprung's disease [25].

Some limitations should be acknowledged. This is a preclinical study and findings have not been confirmed in clinical AKI since that would require a randomized clinical trial. Additionally, whether preservation of tissue protective factors contributes to protection of other organs should be studied, given that Klotho is mainly produced by tubular cells and PGC-1α, as a master regulator of mitochondrial biogenesis, is especially relevant in mitochondria-rich cells such as tubular cells.

Among strengths, the kidney protective role of butyrate and its preservation of kidney protective molecules was demonstrated in two different models of nephrotoxic AKI in both genders as well as in cultured tubular cells. Moreover, different schedules of butyrate administration were explored, and novel therapeutic targets of butyrate, such as Klotho, were identified and validated in independent studies. As discussed above this information may facilitate the clinical translation of the findings through the optimized design of clinical trials.

In conclusion, butyrate protected from AKI and decreased inflammation by preventing the downregulation of kidney protective genes such as Klotho (Fig. 13). Increasing tissue resilience through preservation of tissue protective factors provides a novel mechanistic explanation for tissue protection offered by SCFA, exemplified here by butyrate and AKI. Butyrate inhibited NFκB activation and histone deacetylation, known triggers of the downregulation of factors such as Klotho and PGC-1α in tubular cells, suggesting that the same upstream regulators targeted by butyrate contribute to both decrease inflammatory responses

and preserve kidney protective molecules. The combination of two powerful effects (less inflammation, higher tissue resilience) may explain the success of butyrate therapy in AKI. Eventually, this information should be used to design clinical trials of kidney protection by butyrate.

**Ethics approval:** Procedures were approved by the animal ethics committee of IIS-FJD (PROEX 036/16).

**Author contributions:** MDS-N conceived and designed the experiments. CF and AP-C performed the experiments. CF collected the data and performed statistical interpretation and analysis. CF and MDS-N prepared the Figures. MDS-N, CF and AP-C were responsible for the animal experiment. MDS-N and AO wrote and discussed the manuscript. All authors read and approved the final paper.

**Funding:** European Union's Horizon 2020 Research and Innovation Program (860329 Marie-Curie ITN "STRATEGY-CKD"), FIS/Fondos FEDER (PI22/00469, PI22/00050, PI21/00251, ERA-PerMed-JTC2022 (SPAREKID AC22/00027), Comunidad de Madrid en Biomedicina P2022/BMD-7223, CIFRA\_COR-CM. Instituto de Salud Carlos III (ISCIII) RICORS program to RICORS2040 (RD21/0005/0001) funded by European Union – NextGenerationEU, Mecanismo para la Recuperación y la Resiliencia (MRR) and SPACKDc PMP21/00109. PREVENTCKD Consortium. Project ID: 101,101,220 Programme: EU4H. DG/Agency: HADEA. MDSN and ABS were supported by MICINN Ramon y Cajal program RYC2018-024461-I and RYC2019-026916-I respectively.

**Data Availability:** Data will be made available on request.

**Declaration of competing interest:** All listed authors of this manuscript have approved of the manuscript. AO has received grants from Sanofi and consultancy or speaker fees or travel support from Advicene, Alexion, Astellas, Astrazeneca, Amicus, Amgen, Boehringer Ingelheim, Fresenius Medical Care, GSK, Bayer, Sanofi-Genzyme, Menarini, Mundipharma, Kyowa Kirin, Lilly, Freeline, Idorsia, Chiesi, Otsuka, Novo-Nordisk, Sysmex and Vifor Fresenius Medical Care Renal Pharma and is Director of the Catedra UAM-Astrazeneca of chronic kidney disease and electrolytes. He has stock in Telara Farma.

The rest of the authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

## CRedit authorship contribution statement

**Chiara Favero:** Writing – review & editing, Formal analysis, Data curation. **Aranzazu Pintor-Chocano:** Methodology, Formal analysis, Data curation. **Ana Sanz:** Writing – review & editing. **Alberto Ortiz:** Writing – review & editing, Writing – original draft, Supervision, Resources, Funding acquisition, Conceptualization. **Maria D Sanchez-Niño:** .

## Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: AO has received grants from Sanofi and consultancy or speaker fees or travel support from Advicene, Alexion, Astellas, Astrazeneca, Amicus, Amgen, Boehringer Ingelheim, Fresenius Medical Care, GSK, Bayer, Sanofi-Genzyme, Menarini, Mundipharma, Kyowa Kirin, Lilly, Freeline, Idorsia, Chiesi, Otsuka, Novo-Nordisk, Sysmex and Vifor Fresenius Medical Care Renal Pharma and is Director of the Catedra UAM-Astrazeneca of chronic kidney disease and electrolytes. He has stock in Telara Farma..

## Data availability

Data will be made available on request.

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