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Urinary GM2AP coincides with renal cortical damage and grades cisplatin nephrotoxicity severity in rats

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Abstract:	Nephrotoxicity, including electrolytic disorders and acute kidney injury (AKI), limits the clinical dosage and utility of platinated antineoplastics such as cisplatin. Cisplatin nephrotoxicity embodies a tubulopathy involving the medullary S2 and S3 segments of the proximal and the distal tubules. Higher dosage extends damage over the cortical S1 segment and intensifies overall injury. However, the standard diagnosis based on plasma creatinine as well as novel injury biomarkers lacks enough pathophysiological specificity. Further granularity in the detection of renal injury would help understand the implications of individual damage patterns needed for personalized patient handling. In this article, we studied the association of urinary ganglioside GM2 activator protein (GM2AP) with the patterns of tubular damage produced by 5 and 10 mg/kg cisplatin in rats. Our results show that GM2AP appears in the urine only following damage to the cortical segment of the proximal tubule. The information provided by GM2AP is not redundant with but distinct and complementary to that provided by urinary neutrophil gelatinase-associated lipocalin (NGAL). Similarly, treatment with 150 mg/kg/day gentamicin damages the renal cortex and increases GM2AP urinary excretion; whereas renal ischemia, which does not affect the cortex, has no effect on GM2AP. Because of the key role of the cortical proximal tubule in renal function, we contend GM2AP as a potential diagnostic biomarker to stratify AKI patients according to the underlying damage and follow their evolution and prognosis. Prospectively, urinary GM2AP may help grade the severity of platinated antineoplastic nephrotoxicity by forming part of a non-invasive liquid biopsy.
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Toxicology
Prof. Dr. M. Vinken
Editor-in-Chief

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Dear Prof. Vinken:

We have carefully addressed all the comments made by the Editor and Reviewers to our article titled "Urinary GM2AP coincides with renal cortical damage and grades cisplatin nephrotoxicity severity in rats". We provide an itemized response to all the questions raised and a revised version of our article including the appropriate amendments.

We hope you now find our article interesting for your readers and suitable for publication in Toxicology.

Best regards,

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Reviewer #1:

Authors: The authors thank the reviewer for their detailed revision and suggestions to improve the manuscript, and for making us reconsider some interpretations with a better perspective.

1. "Rats were divided into the following groups (n=3) per group" The authors need to explain why did they choose n= 3.

Authors: The specific n in this study is the result of combining our previous experience with these models and the 3R principles with an aim to minimize the number of animals used to obtaining significant data. Having used these models continuously over the last 20 years has allowed us to optimize experimental conditions and minimize variability.

2. The authors need to add a diagram for the protocol of the experiments.

Authors: Yes, thank you. It is indeed a good idea to add a protocol scheme for clarity's sake and for facilitating reading and comprehension. A new figure (Figure 1) has been added to the article depicting the said scheme, and all figure numbers have been updated accordingly.

3. Figure 1: why did creatinine levels decrease on day 4 when compared to that on day 2 for CP 10 mg/day.

Authors: This must be a misunderstanding. Kindly note that, in fact, plasma creatinine concentration increases from day 2 to day 4 in the CDDP10 group (see top panel in Figure 2, formerly Figure 1).

4. Page 7 results: Result of this study should only be included: Please move results of previous studies to other sections. "We (and others) have previously showed that 5 mg/kg cisplatin causes marked tubular injury restricted to the outer strip of the outer renal medulla (Casanova et al. 2020, 2021; Sancho-Martínez et al. 2020; Fuentes-Calvo et al. 2021; Cuesta et al. 2022a)".

Authors: A similar concept is already presented in the Introduction. In Results, the sentence is more specific with regard to the dose of cisplatin, and we believe it better belongs with this section. It is just an introductory sentence that helps contextualize the following results, without additive value in other sections.

5. How did the authors determine the number of patients in the study?

Authors: A formal population size was not calculated because these experiments were not intended for a protocolized study (which, in the light of the results presented in this study will be the natural continuation), but to substantiate that NGAL and GM2AP do not function in unison, neither in general nor in this specific scenario of patients treated with

cisplatin. There is no further purpose. With this aim, we reused a collection of samples from cisplatin-treated patients, from which we chose those that showed low (or no) urinary level of GM2AP before cisplatin administration.

6. Page 10: "Interestingly, the dose of cisplatin correlates with the increase in urinary GM2AP in oncological patients treated with this drug, in agreement with the findings from the animal model". Table 2 shows 0.456 (0.159). Please explain.

Authors: We are sorry, this was indeed an unfortunate paragraph. Along with the sentence referred to as in the next of the Reviewer's queries (i.e., the number 7), we have reformulated that last paragraph of the Discussion, including the next sentence, alluded in the Reviewer's comment 7.

7. Page 10: "Moreover, the urinary level of GM2AP is independent of Crp, magnesemia and calcemia, indicating that GM2AP bears non-redundant and distinct pathophysiological meaning". Please explain this statement.

Authors: Please, see response to query number 6.

Reviewer #2:

This study entitled "Urinary GM2AP coincides with renal cortical damage and grades cisplatin nephrotoxicity severity in rats" aims to evaluate the use of urinary GM2AP as a biomarker of AKI with cortical but not medullary damage present. This study showed that higher doses of cisplatin extend damage from the medullary region into the cortical tissue, corresponding with an increase in GM2AP. Additionally, urinary GM2AP was not increased 24 hours after unilateral ischemia reperfusion, with damage localized to the medulla. This study provides meaningful insight regarding development of more sensitive biomarkers of AKI to better diagnosis and treat patients in the clinic. Several items could be addressed to improve the strength of this study.

Authors: The authors thank the reviewer for their positive view of our article and for the useful comments inviting to rethink on a couple of respects involving conceptual and description issues, and to make changes neatly improving the manuscript.

Major points:

1. From the results, it is unclear whether GM2AP excretion is more indicative of differences in regional damage or in severity of injury. The I/R model used appeared to induce a much lower level of injury (judged by plasma creatinine and histology scoring) than the cisplatin model used. Evaluation of urinary GM2AP following I/R with a more severe/comparable level of injury to the cisplatin model would be more convincing.

Authors: Our proposal is that GM2AP may potentially serve both for non-invasive regional renal damage introspection and, in the case of platinated antineoplastics, also for injury severity assessment. It is so because, in the case of these drugs, injury spatial patterns coincide with severity, as increasing damage expands from the outer medullary area to the cortical area.

Regarding the extent of damage in the I/R model, every model shows a particular relation among functional and structural injury parameters. For instance, creatinine may increase similarly in two models, one of which showing severe and the other one no parenchymal damage (as in purely pre-renal, hemodynamic models); while in other models intermediate tissue damage scores may be found for the same level of creatinine. On the contrary, models showing similar levels of tissue damage may show different increments in plasma creatinine. This is because there is not a single mechanism inducing the decrease in filtration that translates into the increase in creatinine, so that depending on the mechanisms activated in each model (and the extent to which each of them is activated), filtration becomes more or less compromised (and thus creatinine increases more or less). All in all, the concept of "level of damage" has no absolute but specific meaning: the level of which specific damage. In our model, I/R produces a very similar level of renal medullary damage than gentamicin, despite producing about half the increase in creatinine. But because our article focuses on the relation between cortical damage and urinary excretion of GM2AP, we humbly understand that two models that bear a similar degree of medullary damage, which differ in their affection to the cortical region (regardless of the level of creatinine), serve better purposes in our case than two models equalized by creatinine, with different levels of structural damage.

2. The authors argument that GM2AP is related to S1/S2 injury but not S3 would be strengthened by spatial evaluation of cell death/injury markers in renal tissue. Does urinary GM2AP expression correlate with higher levels of cell death or senescence in the cortex?

Authors: Yes, our data seem to support the relation between urinary GM2AP level and cortical damage level, characterized by tubular cell death. In fact, figure 4 (former figure 3, as we have introduced a new figure -figure 1- depicting the experimental protocol) shows how the dose of cisplatin that causes no (or little) cortical cell death (i.e., 5 mg/kg), shows no increment in GM2AP urinary level; whereas the dose of cisplatin that causes extensive cortical cell death (i.e., 10 mg/kg), also shows a neat increment on GM2AP urinary level.

3. The authors arguments the GM2AP is not related to damage of the S3 segment in the ischemia reperfusion model would be strengthened by a more thorough evaluation of injury in this study. Is cell death/senescence occurring in the medulla or cortex?

Authors: Yes, this is implicitly evaluated in the histological studies, both visually and implicitly in the quantification score, which includes the loss of nuclei (as a surrogate for cell death) as a main injury parameter. Evident tubular necrosis is seen in the affected areas, i.e., the cortex, the outer medulla, or both, depending on the model.

4. Did any of the patients in this study develop AKI by clinical standards after cisplatin dosing? The authors argue that increasing cisplatin dose in rats was directly responsible for elevated urinary GM2AP, however this trend was not observed clinically. What may account for this discrepancy?

Authors: According to internationally recognized criteria (i.e., the KDIGO scale) none of the patients suffered an overt AKI. Regarding the relationship between cisplatin dose and GM2AP urinary level, please, kindly note the following:

Animal models are "cleaner" than human populations with respect to treatment (i.e., human therapies may, and usually, include additional chemotherapeutic drugs or interventions potentially impacting the kidneys) and attendant comorbidities that may modulate (up or down) the effect of cisplatin, resulting in the uncoupling of the cisplatin dose to GM2AP urinary level relationship. In the cases of combined therapies, urinary GM2AP would inform on the composite level of renal cortical affection.

Minor points:

1. All bar graphs should be changed to display individual data points for clarity.

Authors: Following the Reviewer's comment we have changed the graph format as suggested.

2. In Figure 7, displaying the western blots used to measure patient urinary GM2AP would improve transparency.

Authors: GM2AP data shown in figure 8 (former figure 7) is the average of three Western blots carried out with the same samples, quantified, and normalized to urinary creatinine concentration. Accordingly, western blot images do not necessarily correspond to the final, processed data. Anyhow, following the Reviewer's comment, for transparency's sake, we provide a document with the said Western blot images for review purposes.

Urinary GM2AP coincides with renal cortical damage and grades cisplatin nephrotoxicity severity in rats

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Competing Interests. The authors declare no competing interests.

Abbreviations
AKI, acute kidney injury.
BMI, body mass index.
CDDP, cisplatin.
Cl_{Cr}, clearance of creatinine.
Cr_p, plasma creatinine concentration.
Cr_u, urinary creatinine concentration.
DAPI, 4'6-diamidino-2-phenylindole.
ERBP, European Renal Best Practice.
FE_{Glu}, fractional excretion of glucose.
G, gentamicin.
Glu, glucose.
Glu_p, plasma glucose concentration.
Glu_u, urinary glucose concentration.
GFR, glomerular filtration rate.
GM2AP, ganglioside M2 activator protein.
IGFBP7, insulin-like growth factor binding protein 7.
IL-18, interleukin 18.
I/R, ischemia/reperfusion.
KDIGO, Kidney Disease: Improving Global Outcomes
KIM-1, kidney injury molecule 1.
LFABP, liver fatty acid binding protein.
NGAL, neutrophil gelatinase-associated lipocalin.
PSS, physiological saline solution.
TIMP-2, tissue inhibitor of metalloproteinase 2.
UF, urinary flow.

Abstract

Nephrotoxicity, including electrolytic disorders and acute kidney injury (AKI), limits the clinical dosage and utility of platinated antineoplastics such as cisplatin. Cisplatin nephrotoxicity embodies a tubulopathy involving the medullary S2 and S3 segments of the proximal and the distal tubules. Higher dosage extends damage over the cortical S1 segment and intensifies overall injury. However, the standard diagnosis based on plasma creatinine as well as novel injury biomarkers lacks enough pathophysiological specificity. Further granularity in the detection of renal injury would help understand the implications of individual damage patterns needed for personalized patient handling. In this article, we studied the association of urinary ganglioside GM2 activator protein (GM2AP) with the patterns of tubular damage produced by 5 and 10 mg/kg cisplatin in rats. Our results show that GM2AP appears in the urine only following damage to the cortical segment of the proximal tubule. The information provided by GM2AP is not redundant with but distinct and complementary to that provided by urinary neutrophil gelatinase-associated lipocalin (NGAL). Similarly, treatment with 150 mg/kg/day gentamicin damages the renal cortex and increases GM2AP urinary excretion; whereas renal ischemia, which does not affect the cortex, has no effect on GM2AP. Because of the key role of the cortical proximal tubule in renal function, we contend GM2AP as a potential diagnostic biomarker to stratify AKI patients according to the underlying damage and follow their evolution and prognosis. Prospectively, urinary GM2AP may help grade the severity of platinated antineoplastic nephrotoxicity by forming part of a non-invasive liquid biopsy.

Key words: cisplatin, GM2AP, acute kidney injury, acute tubular injury, cortical necrosis, urinary biomarker

1. Introduction

Cisplatin is a widely used platinated chemotherapeutic drug, whose dosage and clinical utility are limited by significant side effects, mainly nephrotoxicity (Sanchez-Gonzlez et al. 2011) ranging from electrolytic disorders to acute kidney injury (AKI) (Blachley and Hill 1981). AKI is an ambiguous term for a multi-etiological and heterogeneous syndrome characterized by a sudden decrease in glomerular filtration rate (GFR) (Basile et al. 2012), with high incidence and derived morbimortality in the short and long term (Doyle and Forni 2016). AKI is especially disastrous for critically ill patients, among whom incidence and mortality escalate to excruciating rates of up to 50% (Ronco et al. 2019). From a pathological perspective, AKI has been traditionally sub classified in pre-renal (i.e., mostly hemodynamic, with no renal parenchymal damage), intrinsic (i.e., derived from primary renal tissue injury, most typically involving the tubule epithelium), and post renal forms (i.e., resulting from obliteration of the urinary ways) (Ronco et al. 2019).

Specifically, cisplatin nephrotoxicity encompasses a tubulopathy affecting the proximal and distal tubules, where the drug mostly accumulates (Sanchez-Gonzlez et al. 2011; Sancho-Martínez et al. 2012). Within the proximal tubule, cisplatin preferentially accumulates and damages the S3 straight segment (Dobyan et al. 1980; Fenoglio et al. 2002; Price et al. 2004; Cristofori et al. 2007; Perše and Večerić-Haler 2018), located mainly in the outer strip of the outer medulla (Zalups et al. 2014). As dosage is increased, the cortical, convoluted S1 and S2 segments also become injured (Jones et al. 1985) resulting in different damage patterns with potentially distinct impact, evolution, and prognosis. Accordingly, precise pathophysiological diagnosis based on the underlying damage pattern is necessary (Moledina and Parikh 2018), for which present diagnostic technology lacks optimal performance. Internationally recognized criteria for AKI diagnosis (the latest of which being the KDIGO and ERBP criteria) rely on increments in plasma creatinine concentration (Cr_p), or on a reduction in urinary output (Fliser et al. 2012; Thomas et al. 2015). Cr_p is an inverse surrogate of GFR with several important limitations, including low sensibility and no specificity on the cause, type, or injury phenotype of AKI (Makris and Spanou 2016). In fact, Cr_p may increase during AKI regardless of the level, and even in the absence of tubular damage (as in pre-renal AKI).

Consequently, Cr_p-blind damage needs to be profiled with new diagnostic methods. In this sense, a generation of so-called *injury biomarkers* (mostly urine-borne) is gaining increasing attention and slowly erupting into the clinical scenario, for their association with renal injury independently, and even in the absence, of increments in Cr_p. These biomarkers most typically include kidney injury molecule 1 (KIM-1), neutrophil gelatinase-associated lipocalin (NGAL), interleukin 18 (IL-18), liver fatty acid binding protein (LFABP), tissue inhibitor of metalloproteinase 2 (TIMP-2) and insulin-like growth factor binding protein 7 (IGFBP7) (Vanmassenhove et al. 2019). However, the definition, classification, and diagnosis of subclinical AKI by means of "injury biomarkers" is still a quite vague concept. Patients positive for a specific biomarker may be, in turn, negative for another. This suggests that each biomarker has a particular biological and diagnostic meaning arising from a yet undetermined but distinctive damage pattern or specific pathophysiological process. Thus, further investigation and knowledge on their etiology, origin, and traffic among biological fluids and compartments is needed to endow these biomarkers with net clinical significance and utility.

With this aim, this article focuses on ganglioside GM2 activator protein (GM2AP), a novel and sensitive injury biomarker candidate whose urinary level has been previously related to AKI. GM2AP increases in the urine of different animal models of toxic AKI (Sancho-Martínez et al. 2022). Urinary GM2AP shows high sensitivity and early diagnostic capacity. Indeed, GM2AP appears in the urine as a consequence of defective tubular reabsorption, even under subclinical AKI conditions (i.e., normal Cr_p), and largely precedes Cr_p elevation during overt AKI (Quiros et al. 2010). GM2AP also increases in the urine of patients undergoing AKI in different clinical contexts (Blanco-Gozalo et al. 2020; Vicente-Vicente et al. 2021), and contributes to anticipating delayed recovery and bad prognosis (Blanco-Gozalo et al. 2020).

In this article, we provide further pathophysiological contextualization of urinary GM2AP as a marker of renal cortical damage allowing for gradation of cisplatin nephrotoxicity severity.

2. Materials and Methods

Where not otherwise indicated, reagents were purchased from Sigma-Aldrich.

ARRIVE Statement

Animal experiments were carried out in compliance of ARRIVE Guidelines.

2.1. Animals and experimental protocol

All procedures were approved by the Bioethics Committee of the University of Salamanca and the Regional Government of Castile and Leon, Ministry of Agriculture and Livestock. Animals were handled according to the guidelines of the European Community Council Directive 2010/63/UE, and to the current Spanish legislation for experimental animal use and care (RD 53/2013). Male Wistar rats (220−240 g; Charles River, Madrid, Spain) were used and maintained under controlled environmental conditions within the University of Salamanca Animal House facility, with free access to water and standard chow. Four types of experiments were performed (Figure 1):

- Experiment 1: rats were divided into the following groups (n=3) per group: (i) Control: rats received physiological saline solution (PSS, the vehicle) i.p.; (ii) Cisplatin-5 (CDDP5): a single i.p. injection of 5 mg/kg cisplatin; (iii) Cisplatin-10 (CDDP10): a single i.p. injection of 10 mg/kg cisplatin. Animals were followed for 4 days. In these rats, renal function (section 2.2.) and renal histology (section 2.3.) were assessed as described below. For these purposes, rats were allocated in metabolic cages at specific time points for individual urine collection. Urine was cleared by centrifugation and stored at -80 ºC. Blood was obtained from a small incision in the tail vein and immediately centrifuged to obtain the plasma, which was kept at -80 ºC until use. At day 4, rats were anesthetized with 80 mg/kg ketamine + 20 mg/kg xylazine and their kidneys perfused with saline solution to remove the blood, dissected and immediately fixed in 3,7% p-formaldehyde for histological studies.
- Experiment 2: in a separate set of experiments, rats (n=3 per group) were treated as above and, 4 days after cisplatin treatment, *in situ* renal cell viability studies were performed as described below (section 2.4.).
- Experiment 3: in a third set of experiments, rats (n=3 per group) were treated as above and, 4 days after cisplatin treatment, were anesthetized (as above) and the jugular vein and the urinary bladder were cannulated. A single injection of sodium maleate (400 mg/kg), a proximal tubule reabsorption inhibitor (Christensen and Maunsbach 1980;

Cho et al. 1985; Hysting et al. 1987; Uwai and Nabekura 2021), was administered through the jugular catheter. Urine was collected from the bladder catheter during 30 minutes before maleate administration, and for 90 minutes thereafter, centrifuged, and kept at -80 ºC.

- Experiment 4: in a fourth set of experiments, rats (n=3 per group) were divided into the following groups: (i) Control: rats received physiological saline solution (PSS, the vehicle) i.p.; (ii) Gentamicin (G): rats received 6 daily, consecutive i.p. injections of 150 mg/kg gentamicin; (iii) Ischemia (I/R): Renal ischemia/reperfusion was carried out basically as described (Paniagua-Sancho et al. 2021). Briefly, rats were anesthetized with a mixture of 80 mg/kg ketamine and 20 mg/kg xylazine. Kidneys were accessed through a medial laparotomy and the left renal artery gently exposed, cleaned from surrounding fat, and clamped for 60 minutes, after which the clamp was removed to allow for reperfusion. The right hilum was ligated, and the kidney was dissected. Incisions were then sutured, and animals were allowed to recover under 30 μg/kg buprenorphine analgesia. 24 hours after the ischemic procedure (in the I/R group) and 7 days after inception of the gentamicin regime (in the G group), blood samples, and renal tissue specimens were obtained, processed, and kept as described above. Cr_p was measured (as indicated in section 2.2.), and histological assessment was performed on renal specimens as described in section 2.3.

In all cases, GM2AP was measured in the urine as described in section 2.5.

2.2. Renal function analysis

GFR was monitored by means of Cr_p and creatinine clearance (Cl_{Cr}). Cl_{Cr} was calculated using the equation $Cl_{Cr} = (Cr_u \times UF)/ Cr_p$, where Cr_u stands for the urinary concentration of creatinine, and UF for urinary flow. Cr_p and Cr_u were measured with a commercial, colorimetric assay based on the Jaffe reaction (*Quantichrom Creatinine Assay Kit*, BioAssay Systems, Hayward, CA, USA). Tubular performance was assessed by means of the fractional excretion of glucose (FE_{Glu}), defined as $(Glu_u \times Cr_p) / (Glu_p \times Cr_u)$, where Glu_u and Glu_p stand for the concentration of glucose in the urine and plasma, respectively. Glu_u and Glu_p were measured with commercial reactive strips (Bayer, Leverkusen, Germany).

2.3. Renal tissue assessment

For histopathological studies, paraffin blocks were made with 3.7% p-formaldehyde-fixed kidneys, and 5-μm tissue sections were stained with hematoxylin and eosin for visual inspection under the microscope. Renal injury was blindly determined by means of a "severity score", which was calculated semi quantitatively as described (14). A score of 0–3 was assigned to each section, according to the following criteria: 0, normal histology; 1, tubular cell swelling, brush border loss, nuclear condensation, or loss of nuclei in up to one third of the tubular perimeter; 2, same as for 1, but from one third to two third of nuclei loss; and 3, over one third of nuclei loss. Section scores were added to give a field score (maximal score per field = 30). The average score of 10 fields was used for each kidney sample.

2.4. *In situ* evaluation of renal cell viability with ethidium homodimer

Ethidium homodimer is a cell impermeable, DNA-binding, fluorescent dye excluded from viable cells, used for *in situ* cell viability studies (Edwards et al. 2007, 2020; Prozialeck et al. 2009). For this purpose, on day 4, animals were anesthetized with 80 mg/kg ketamine + 20 mg/kg xylazine. A laparotomy was performed to expose the left

kidney, which was perfused through a catheter placed in the aorta after ligation of the right renal and main mesenteric arteries, and the aorta right above and below the catheter. Perfusion was carried out with 5 µM ethidium homodimer in PSS for 5 minutes at a 1 mL/min flow, then for another 5 minutes at 3 mL/min and, finally, with PSS at 3 mL/min for 10 minutes (to eliminate the excess ethidium homodimer). Perfusion pressure was monitored during the whole process with a pressure transducer connected to a MP150 digital data recorder (Biopac Systems, Goleta, CA, USA), and verified to not exceed 100 mmHg. After perfusion, the left kidney was dissected, and cut longitudinally into two halves. One was fixed in 3,7% paraformaldehyde and used for histological studies (as described in the next section), and the other half was preserved in 30% saccharose in PBS (138 mM NaCl, 2.6 mM H_2KPO_4, 4.1 mM Na_2HPO_4) for fluorescence visualization. These specimens were fixed with ice-cold methanol, embedded in paraffin, cut into 5 µm slices, placed on glass slides, stained with 1 mg/mL 4'6-diamidino-2-phenylindole (DAPI), preserved with DPX mounting medium under coverslips and visualized and photographed under an Axiovert 200M inverted microscope (Zeiss, Madrid, Spain). To quantify cortical necrosis, photographs of 10 cortical fields per specimen were evaluated. Cortical necrosis was evaluated as the average % of ethidium homodimer-positive cells in the 10 fields, out of all cells (i.e., ethidium homodimer-positive + DAPI-positive cells).

2.5. Urinary GM2AP measurement

Western blot was used to measure urinary GM2AP. Western blot was run with a volume of urine from each animal corresponding to the same fraction of urinary output. Urine samples were separated by acrylamide electrophoresis. Proteins were transferred to an Immobilon-P Transfer Membrane (Millipore, Madrid, Spain) and incubated with anti-GM2AP polyclonal antibody produced for our laboratory (by Immunostep, Salamanca, Spain) as described (10), followed by a horseradish peroxidase-conjugated goat anti-rabbit secondary antibody (Southern Biotech, Birmingham, AL, USA). A positive control was used in all experiments to normalize biomarker levels, and all biomarkers were expressed as arbitrary units (% of positive control, which was assigned the 100% value). Bands were quantified and normalized to the intensity of the positive control. The positive control was urine from a highly proteinuric rat urine obtained and tested positive for GM2AP in pilot studies.

2.6. Patients and clinical protocol

An observational study, designed as proof-of-concept, was conducted to pre-emptively evaluate the change in GM2AP urinary level in a clinical scenario with cancer patients from the Medical Oncology Service of the University Hospital of Salamanca (Salamanca, Spain) following treatment with the antineoplastic drug cisplatin. The study fulfilled the principles established in the accordance with the Declaration of Helsinki (World Medical Assembly), the Council of Europe Convention on Human Rights and Biomedicine, the UNESCO Universal Declaration on the Human Genome and Human Rights, the requirements established in the Spanish legislation in the field of biomedical research, personal data protection and bioethics; as well as the provisions of the Law 14/2007, of July 3rd, of Biomedical Research; and RD 53/2013, of February1st. The study was approved by the University Hospital of Salamanca Ethical Committee and all patients provided written consent. The following information was collected from each patient: sex, age, weight, height, body mass index (BMI), body surface area and cisplatin dose. Urine and blood samples were collected immediately before and 72 hours after cisplatin administration. The following analytical data were extracted from their medical records before and after the first chemotherapy cycle: plasma creatinine, plasma urea, calcemia and magnesemia. GM2AP and NGAL were measured in urine samples. GM2AP was

determined as described above, but in this case 21 μL urine from each patient were used. NGAL was measured with a commercial ELISA (Human NGAL ELISA Kit 036CE, BioPorto Diagnostics, Hellerup, Denmark) following the manufacturer's instructions. Both GM2AP and NGAL data were normalized by the corresponding urinary creatinine concentration (Cr_u) in each sample.

2.7. Statistical analysis

Data normality was evaluated with the Shapiro–Wilk test. For data with a normal distribution, two-way ANOVA was applied and the Dunnett´s or Tukey´s post hoc tests were used for multiple and simple comparisons. For non-normal data, Kruskal-Wallis and Dunn´s tests were used. On the other hand, Pearson's correlation tests (for normal data) or Spearman's (for non-normal data) were applied to evaluate the association among patient data parameters. In all cases, statistical significance was established at $p < 0.05$. Statistical analysis was performed with the GraphPad Prism 7.0 software (GraphPad Software, San Diego, CA, USA).

3. Results

3.1. Increasing cisplatin dosages produce increasing cortical tubular necrosis despite causing a similar level of AKI

We (and others) have previously showed that 5 mg/kg cisplatin causes marked tubular injury restricted to the outer strip of the outer renal medulla (Casanova et al. 2020, 2021; Sancho-Martínez et al. 2020; Fuentes-Calvo et al. 2021; Cuesta et al. 2022a). In pilot studies we realized that a higher dose of cisplatin (10 mg/kg) caused renal parenchymal damage extending to the renal cortex in rats. Collectively, Figures 2, 3 and 4 show that despite causing an almost identical level of AKI, as measured by the international criterium (i.e., plasma creatinine concentration), and almost identical renal medullary affection, 10 mg/kg cisplatin caused a marked cortical necrosis that is absent following administration of 5 mg/kg cisplatin. Figure 2a shows that both dosages of cisplatin cause a similar increment in the gold standard AKI diagnostic parameter, Cr_p, and a similar reduction in GFR measured as clearance of creatinine. However, more extensive proximal tubular affection is evident after 10 mg/kg than after 5 mg/kg cisplatin from the higher fractional excretion of glucose (Figure 2b), and the cortical damage observed in the histological study. Figure 3 depicts representative images of both the cortical and the outer medullary areas of rats treated with no (controls), 5 and 10 mg/kg cisplatin. For a similar medullary involvement, 10 mg/kg cisplatin produces marked cortical damage characterized by extensive tubular necrosis, which is not observed in the other groups. *In vivo* labelling of necrotic cells with ethidium homodimer shows very similar results (Figure 4). Whereas substantial necrosis is detected after 10 mg/kg cisplatin in the cortical area, only a low level is seen after 5 mg/kg.

3.2. GM2AP appears in the urine coinciding with cortical injury and after inhibition of proximal tubule reabsorption

Interestingly, the tubular injury marker GM2AP is elevated in the urine only when rats are treated with 10 mg/kg and not (or minimally) with 5 mg/kg cisplatin, compared with controls (Figure 5a). As a corollary, the AKI group bearing cortical damage excretes GM2AP in the urine, whereas the AKI group not showing cortical involvement does not. In addition, inhibition of proximal tubule transport with sodium maleate in normal rats also caused an increase in the urinary excretion of GM2AP (Figure 5b), showing a potential mechanism explaining GM2AP urinary excretion in rats with damaged proximal

tubules. Moreover, an additional insult causing renal cortical damage (i.e., the aminoglycoside antibiotic gentamicin) also shows increased urinary excretion of GM2AP; whereas I/R, which produces no harm to the renal cortex, does not (Figures 6 and 7).

3.3. GM2AP in patients treated with cisplatin

With an aim to gaining a first, pilot view on whether GM2AP is also excreted in the urine of cisplatin-treated patients, we studied a group of cancer patients to be treated with this drug who did not excrete GM2AP prior to the oncological therapy (or had only minimal excretion) and who were otherwise unselected according to any other characteristics. If GM2AP behaved in humans according to the experimental model, patients might show increased or unaltered GM2AP urinary excretion following cisplatin administration, depending on the degree of proximal tubule affection. Patient characteristics are depicted in Table 1. As shown in Figure 8, urinary GM2AP was not increased by cisplatin therapy in patients # 2, 6, 8 and 10, whereas it was elevated in patients 1, 3, 4, 5, 7, 9 and 11 to variable degrees after cisplatin administration. The pathophysiological (and potential diagnostic) information provided by urinary GM2AP is not redundant but complementary to that drawn from another tubular injury biomarker, i.e., NGAL, which had a distinctive urinary excretion pattern. In fact, the incremental excretion of GM2AP (i.e., the difference between the excretion prior to cisplatin and that observed after cisplatin) showed no correlation with any other parameter related to the impact of cisplatin on renal function and integrity (Table 2).

4. Discussion

Two critical elements of a prospective pathophysiological diagnosis are the type of damage and the tubular segments involved. To this end, this article provides new information on the meaning of urinary GM2AP on proximal tubule pathophysiology. The proximal tubule is a highly susceptible part of the nephron and, by reabsorbing the majority of the ultrafiltrate, a determinant of overall nephron function. Proximal S1, S2, and S3 segments have distinct anatomical, histological, biochemical, and functional characteristics, and differential susceptibility to injury and to specific insults (Venkatachalam et al. 1978; Christensen et al. 2021). In fact, the S3 has been proposed to provide functional reservoir for S1 and S2. When reabsorption in S1 and S2 is overwhelmed, the S3 is recruited (Christensen et al. 2021). However, the impact of damage to each proximal tubule segment on disease progression and outcome remains unknown due to the absence of sufficiently granular diagnosis. Our results show that the increased urinary excretion of GM2AP occurs in coincidence with the dosages of cisplatin that produce cortical tubular damage and thus unveil GM2AP as a potential candidate biomarker of damage to the S1 and S2 segments.

The dose-dependent cortical damage induced by cisplatin is recapitulated in our experimental model. It is not until the S1 and S2 segments become damaged that GM2AP urinary excretion is increased. In agreement, urinary GM2AP was previously shown to increase also in a rat model of gentamicin nephrotoxicity (Quiros et al. 2010), a drug known to affect the S1 and S2 segments of the proximal tubule (Wedeen et al. 1983; Zhang et al. 2009; Lopez-Novoa et al. 2011). GM2AP seems to detect a wide range of S1/S2 damage levels, ranging from subtle alterations in the reabsorption machinery (such as in gentamicin-induced predisposition to AKI or following exposure to maleate (Quiros et al. 2010)), to overt S1/S2 necrosis (i.e., as induced by gentamicin or high dose

cisplatin), in which tubules are incapable of normally reabsorbing the filtered GM2AP (Quiros et al. 2010).

Identification of the type of damage (i.e., from sublethal, functional alterations to tubular necrosis) is of critical diagnostic interest since unrepaired damage has direct impact on outcome. Specifically, AKI involving parenchymal damage associates with worse evolution and prognosis (Liaño et al. 1996; Esson and Schrier 2002; Bellomo et al. 2012; Uchino et al. 2012; Rachoin et al. 2012; Endre et al. 2013; Yang et al. 2014; Sawhney et al. 2015; Barasch et al. 2017). In fact, damaged tubules have a remarkable but limited capacity of repair. Following an AKI, proximal tubules regenerate but only to a shorter length, with repeated AKI episodes accumulating further reductions (Endo et al. 2015). Together with irreversible nephron loss (Kellum et al. 2021) and persisting tubular atrophy with dedifferentiated tubule cells stagnated in a profibrotic phenotype (Bonventre 2014; Venkatachalam et al. 2015), tubule shortening contributes to maladaptive repair and progression to chronic kidney disease (Ferenbach and Bonventre 2015; Venkatachalam et al. 2015). In fact, repeated AKI has been shown to independently provide cumulative risk for developing CKD (Thakar et al. 2011). In the short term, sub-clinical renal tissue sequelae (i.e., persisting after Cr_p normalization) (Fuentes-Calvo et al. 2021; Cuesta et al. 2022b) correlate with abnormally increased and remaining susceptibility to new AKI episodes (Fuentes-Calvo et al. 2021).

Because urinary GM2AP provides segment specificity but not information on the type of damage (i.e., potentially ranging from sublethal, functional alteration to overt tubular necrosis), complementary biomarkers are needed to contextualize its precise meaning in each AKI case. *Injury biomarkers* pose immediately obvious candidates. However, the pathophysiological meaning of most (if not all) *injury biomarkers* is still incompletely understood. For example, NGAL, TIMP-2 and IGFBP7 were long regarded as markers of tubular injury directly shed to the urine by damaged tubules. Nevertheless, recent studies (Johnson and Zager 2018; Sancho-Martínez et al. 2020; Skrypnyk et al. 2020) have shown that their increased urinary excretion following acute renal injury is mostly (and probably only) due to defective tubular reabsorption. These findings extend their diagnostic sensitivity to milder (i.e., sub-lethal) and even subclinical forms of damage, but reduce their specificity. An increased urinary excretion of these injury biomarkers may be as reflective of subtle alterations in tubular function as of overt tubular necrosis. This is further complicated by the ambiguous relation of the extent of structural tubular damage and the urinary level of injury biomarkers (Sancho-Martínez et al. 2022), an effect that may be partly explained by sub-lethal alterations impacting biomarker reclamation. Consequently, an unmet diagnostic need and a challenge for the future is to identify urine-borne, segment-specific biomarkers of tubule cell death and tissue derangement.

Our results also support that, rather than redundant, injury biomarkers seem to be mostly complementary. In fact, the excretion pattern of GM2AP in the unselected group of patients included in this study has no similitude with that of NGAL. Injury biomarkers do not function in unison and, thus, cannot be used collectively as a class, as frequently proposed. In fact, a redefinition of AKI including injury biomarkers is increasingly suggested, which proposes three diagnostic phenotypes as per increments in either Cr_p or injury biomarkers, or in both (Moledina and Parikh 2018; Desanti De Oliveira et al. 2019; Ronco et al. 2019; Ostermann et al. 2020). Our results support that, on the contrary, injury biomarkers must be used according to their specific biological meaning as parts of additive fingerprints.

GM2AP may be proposed as a candidate marker to monitor the extent of tubular damage and the severity of cisplatin nephrotoxicity and, also, of subclinical nephrotoxicity, as its urinary levels increase in specific patients in whom no increments in Cr_p are evidenced. Indeed, tubular alterations occurring with normal Cr_p, are typical of therapeutic courses with platinated antineoplastics, resulting in hypomagnesemia and hypocalcemia (Casanova et al. 2020). Interestingly, GM2AP contributes supplemental pathological information on tubular injury, which is blind to these electrolytic disturbances, as low and insignificant correlations exist between them.

Moreover, GM2AP might provide additional diagnostic utilities. For instance, the relation between cisplatin dose and injury extension seen in animal models may be partially distorted or uncoupled in patients by concomitant therapies frequently used in combined anticancer therapies. In these circumstances, a liquid biopsy (potentially including GM2AP) may better profile the actual renal damage inflicted by the specific treatment in each individual patient, for a personalized handling with precision medicine criteria. Regulated and structured clinical studies involving appropriate cohort sizes are now necessary to deeply understand the incremental diagnostic value of urinary GM2AP on the limitations of traditional clinical parameters.

5. Conclusions

In summary, or study unveils urinary GM2AP as a candidate biomarker of damage to the renal proximal tubule S1 and S2 segments with subclinical sensitivity, contributing increased granularity to a prospective pathophysiological diagnosis of AKI. Furthermore, in the case of treatments with platinated antineoplastics such as cisplatin, the presence of GM2AP in the urine may be indicative of more severe parenchymal damage extending to the cortical area.

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Table 1. Patient characteristics. BMI, body-mass index. ND, not determined. Post, 72 h after cisplatin. Pre, before cisplatin administration.

Patient	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**
Sex	Male	Male	Female	Male	Male	Female	Male	Male	Male	Female	Male
Age (years)	45	53	68	54	62	41	43	60	64	53	52
Weight (kg)	103	81	70	84	71	61	90	60	65	68	75
Height (cm)	177	165	148	170	165	148	181	173	163	159	175
BMI (kg/m^2)	32.9	29.8	32.0	29.1	25.9	27.8	27.5	20.0	24.5	26.9	24.5
Body Surface (m^2)	2.2	1.9	1.6	1.9	1.8	1.5	2.0	1.8	1.7	1.8	1.9
Cisplatin dose (mg)	150	110	80	47	140	110	150	135	120	100	135
Plasma creatinine Pre/Post (mg/dL)	0.95/ 1.04	0.72/ND	0.70/ND	1.16/ 1.17	0.65/ND	0.72/ 0.66	1.34/ 0.76	0.98/ 1.11	0.80/ 0.71	0.71/ND	0.74/ 0.76
Plasma urea Pre/Post (mg/dL)	40/ 50	35/ND	32/ND	49/ 49	40/ND	20/ 19	71/ 42	38/ 56	30/ 22	25/ND	37/ 35
Calcemia Pre/Post (mg/dL)	8.8/ 8.4	9.5/ND	9.8/ND	11.0/ 9.9	8.8/ND	8.9/ 8.4	9.1/ 8.3	9.7/ 7.5	9.5/ 8.8	9.5/ND	9.6/ 9.4
Magnesemia Pre/Post (mg/dL)	2.1/ 2.1	ND/ND	1.8/ND	1.5/ 1.8	2.3/ND	1.8/ 2.2	2.0/ 2.4	2.2/ 1.8	2.1/ 2.4	2.0/ND	1.9/ 2.0

Table 2. Correlation between cisplatin dose and biomarker level in patients. Data represent Pearson's R coefficient (p-value) for normal data or Spearman's rho coefficient (p-value) for non-normal data. Δ, increment from immediately before cisplatin administration to 72 hours afterwards. *, p<0.05; **, p<0.01.

	Cisplatin dose	Δ Plasma creatinine	Δ Plasma urea	Δ Calcemia	Δ Magnesemia	Δ NGAL	Δ GM2AP
Cisplatin dose		0.127 (0.786)	-0.110 (0.814)	0.115 (0.807)	-0.338 (0.458)	0.207 (0.556)	0.456 (0.159)
Δ Plasma creatinine			0.893 (0.007)**	0.000 (1.000)	-0.821 (0.023)*	0.250 (0.589)	0.643 (0.119)
Δ Plasma urea				-0.406 (0.365)	-0.779 (0.039)*	0.131 (0.780)	0.474 (0.282)
Δ Calcemia					0.633 (0.127)	-0.464 (0.295)	0.100 (0.831)
Δ Magnesemia						-0.611 (0.145)	-0.525 (0.227)
Δ NGAL							0.146 (0.687)
Δ GM2AP							

Figures

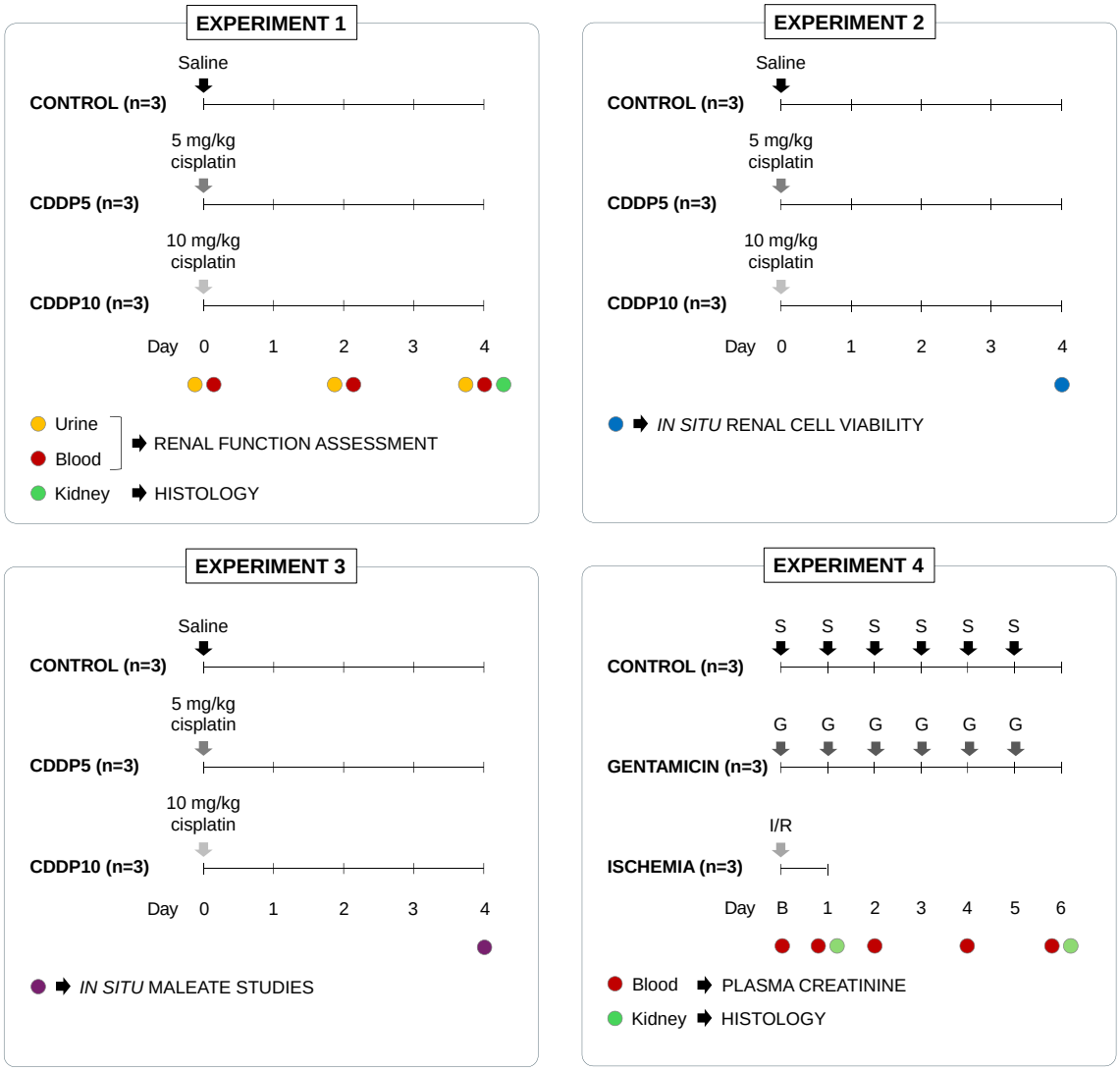

Figure 1. Schematic representation of the experimental protocols used in this study. B, basal. CDDP, cisplatin. G, gentamicin. I/R, ischemia/reperfusion. S, saline.

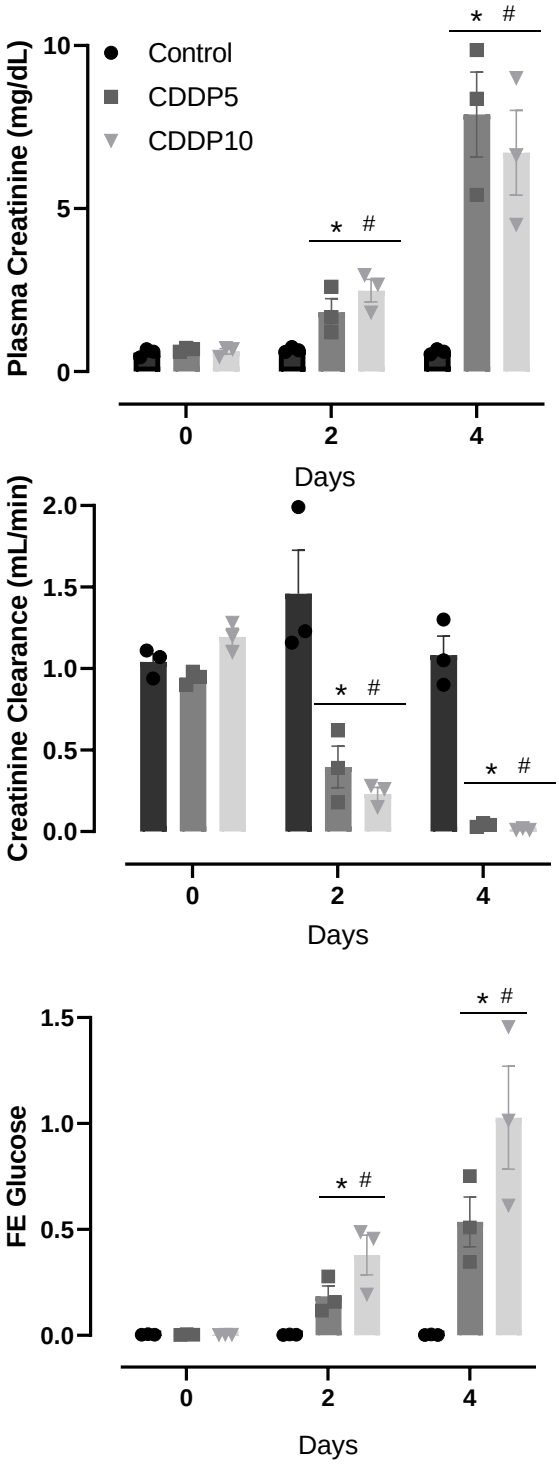

Figure 2. Renal function assessment following treatment with cisplatin. (a) Plasma creatinine concentration; **(b)** creatinine clearance; **(c)** fractional excretion (FE) of glucose, from rats treated with 5 or 10 mg/kg cisplatin. Data are expressed as the mean ± SEM of n=3 per group. *, $p<0.05$ vs the control (on the same day); and #, $p<0.05$ vs. day 0 in the same group. CDDP, cisplatin.

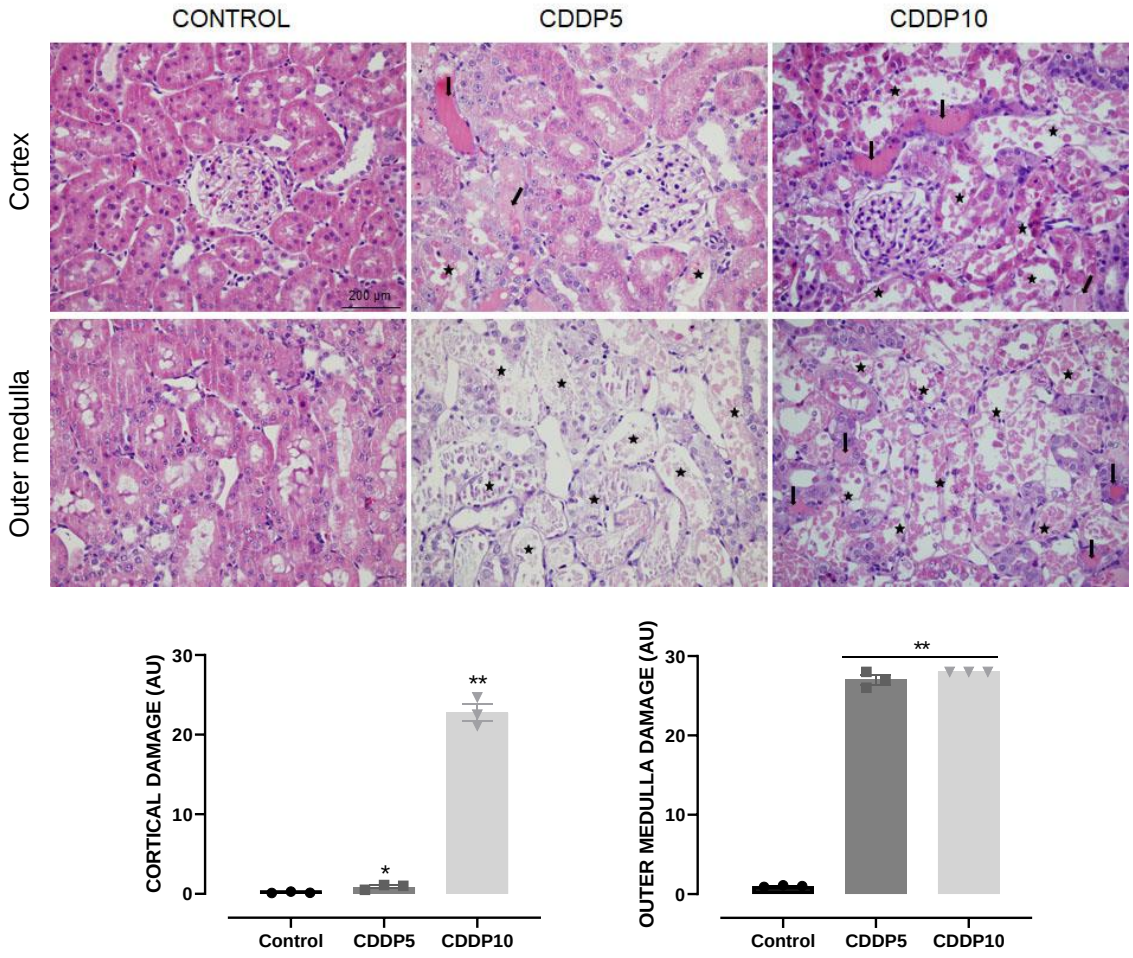

Figure 3. Renal tissue damage patterns produced by cisplatin. Upper panel show representative images (400x) of the cortical and outer medullary areas of renal sections from rats treated with 5 (CDDP5) or 10 mg/kg (CDDP10) cisplatin, stained with hematoxylin-eosin, showing tubular necrosis with tissue debris (*) and tubular obstruction (↓). The lower panels depict the corresponding damage scoring. Data are expressed as the mean ± SEM of n=3 per group. *, $p<0.05$ and **. $p<0.01$ vs the control. AU, arbitrary units.

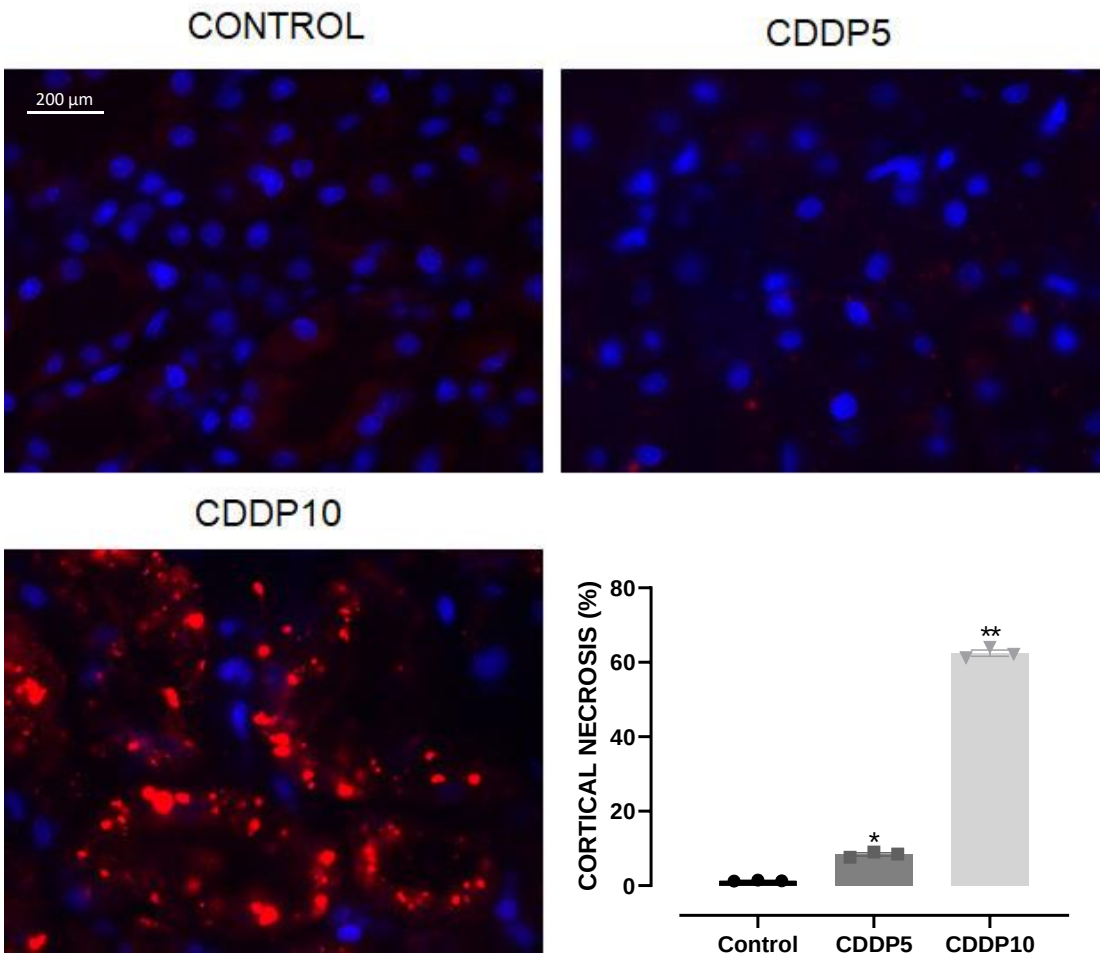

Figure 4. Cortical necrosis assessment following treatment with cisplatin. Representative images (400x) of the cortical area of renal sections from rats treated with 5 or 10 mg/kg cisplatin stained with ethidium homodimer, and the corresponding scoring (% of necrotic cells). Data are expressed as the mean ± SEM of n=3 per group. *, $p<0.05$. **, $p<0.01$ vs the control. CDDP, cisplatin.

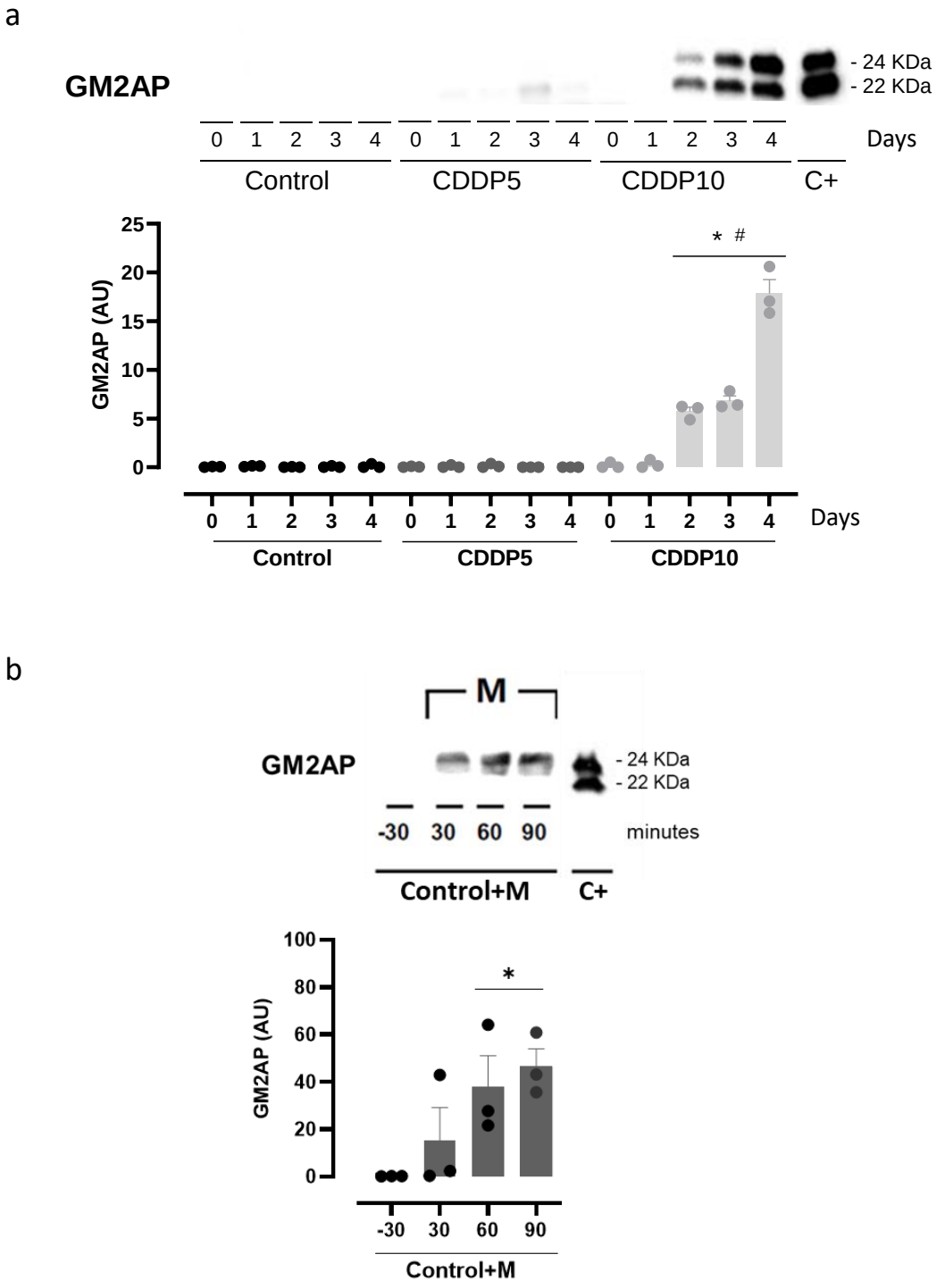

Figure 5. Urinary excretion of GM2AP caused by cisplatin. a) GM2AP appears in the urine following treatment with 10 but not with 5 mg/kg cisplatin. **b)** Effect of sodium maleate on GM2AP urinary excretion. Representative image of Western blot analysis for urinary GM2AP (upper panel), and the corresponding densitometric quantification (lower panel)**.** Data are expressed as the mean ± SEM of n=3 per group. *p<0.05 vs the control (on the same day); and #p<0.05 vs. day 0 in the same group. AU, arbitrary units. CDDP, cisplatin. C+, positive control. M, maleate.

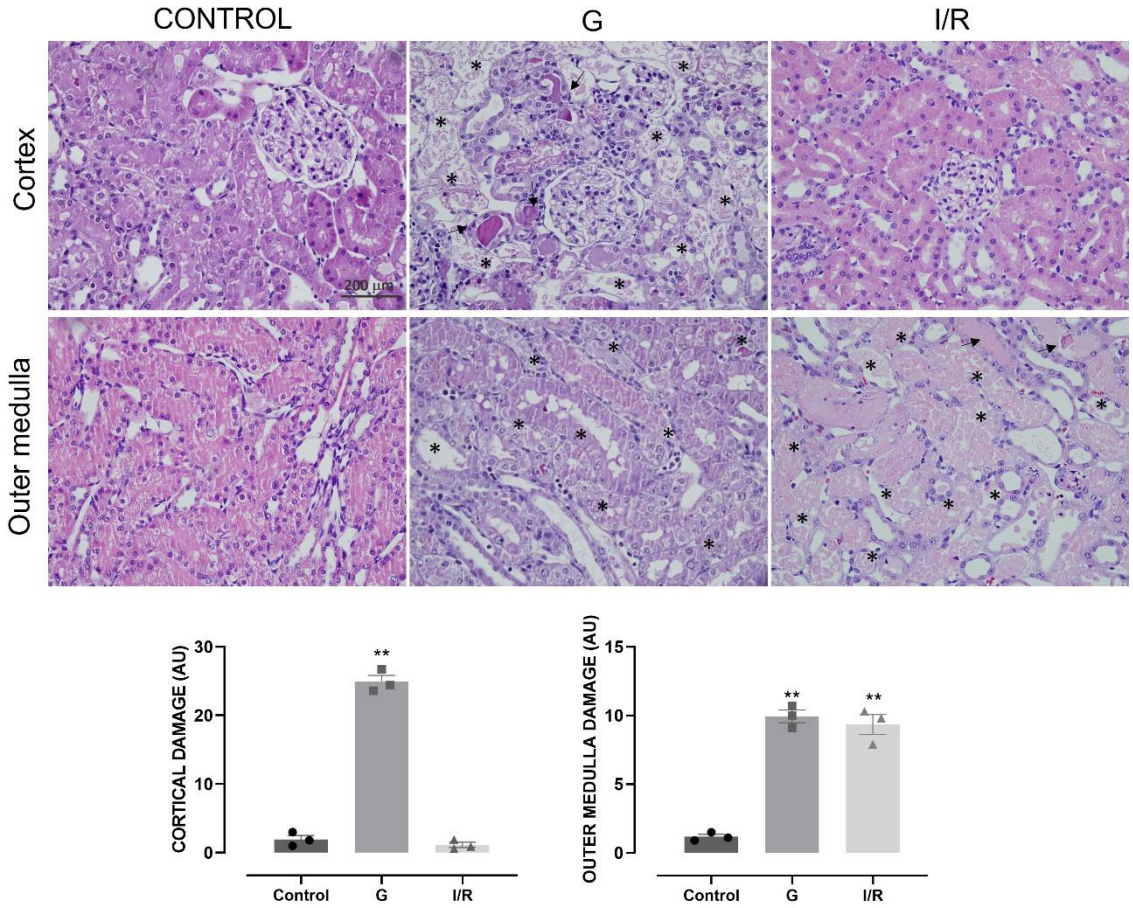

Figure 6. Renal tissue damage patterns produced by gentamicin and ischemia/reperfusion. Upper panel show representative images (400x) of the cortical and outer medullary areas of renal sections from rats treated with gentamicin (G) or subjected to renal ischemia/reperfusion (I/R), stained with hematoxylin-eosin, showing tubular necrosis with tissue debris (*) and tubular obstruction (↓). The lower panels depict the corresponding damage scoring. Data are expressed as the mean ± SEM of n=3 per group. **. $p<0.01$ vs the control. AU, arbitrary units.

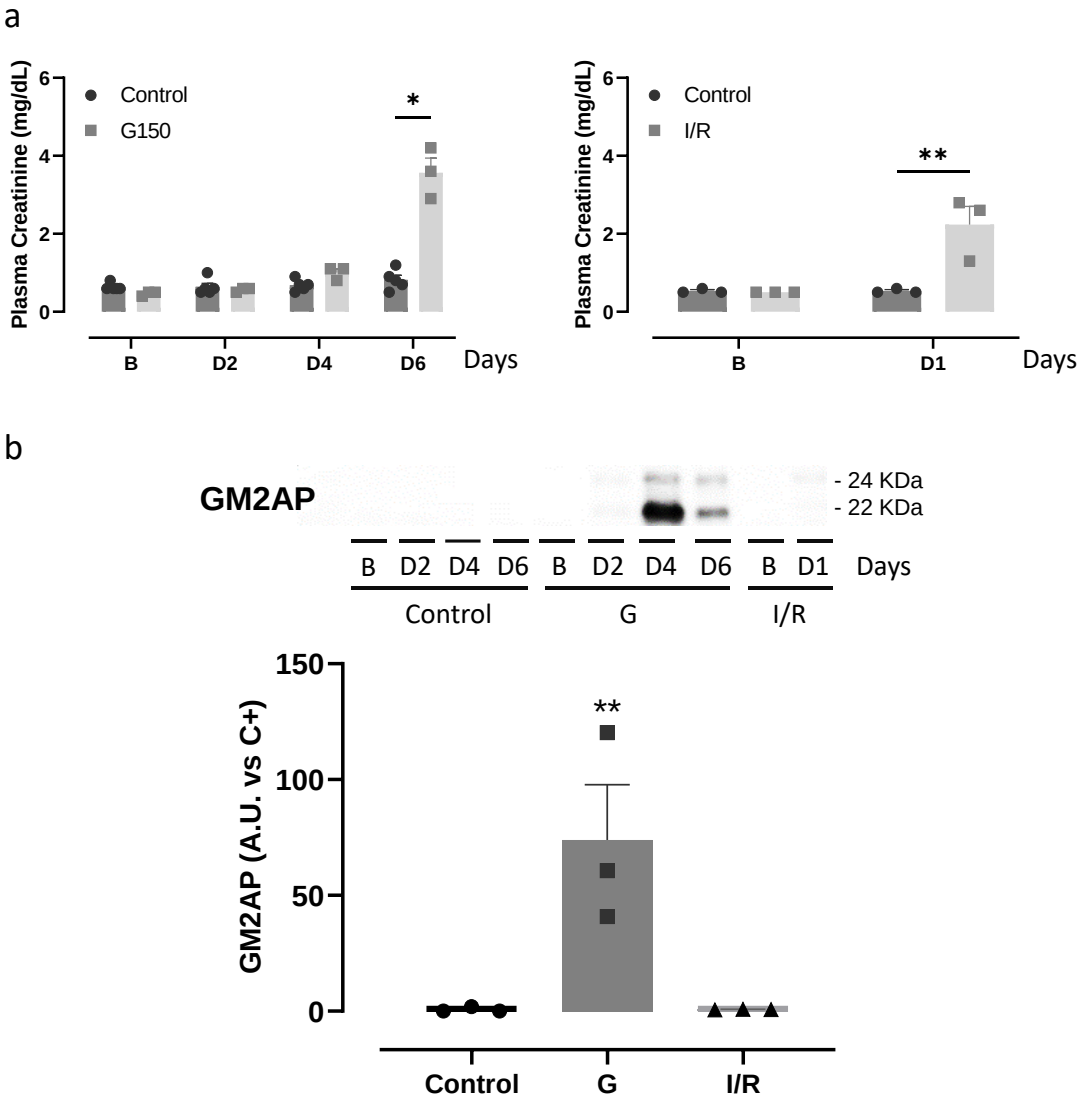

Figure 7. Renal function and urinary excretion of GM2AP following treatment with gentamicin and ischemia/reperfusion injury. (a) Plasma creatinine concentration; **(b)** urinary excretion of GM2AP of rats treated with gentamicin (G) or subjected to renal ischemia/reperfusion (I/R). Data are expressed as the mean ± SEM of n=3 per group. *, p<0.05, **. p<0.01, ***, p<0,001 vs the control. AU, arbitrary units.

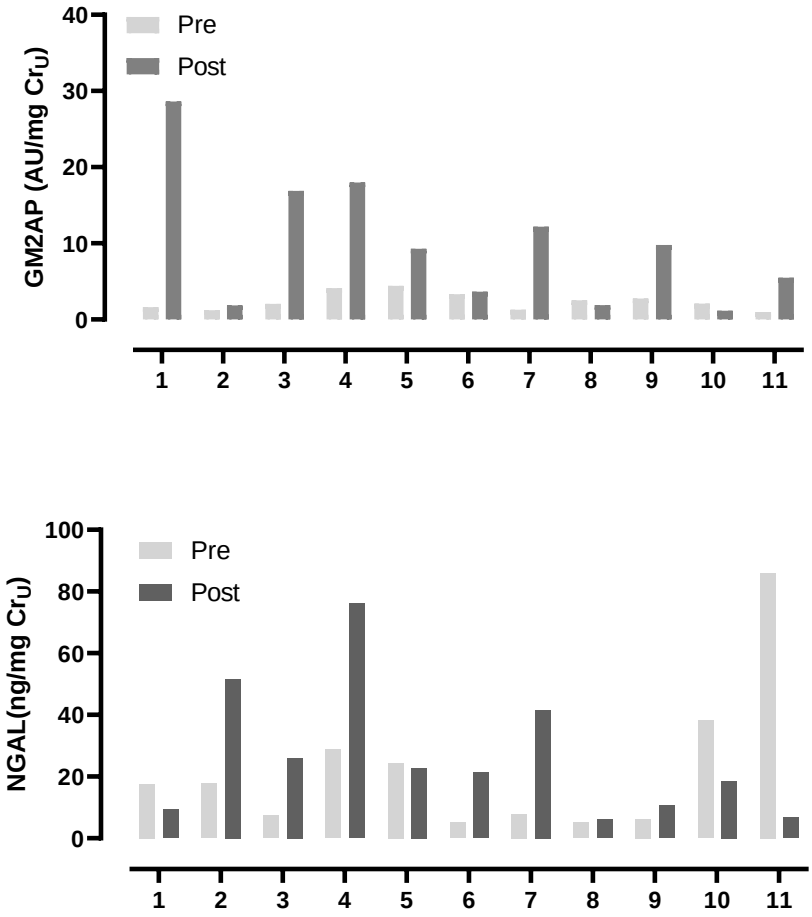

Figure 8. Profile of GM2AP and NGAL urinary excretion for 11 patients before (Pre) and 72 hours after (Post) cisplatin administration. AU, arbitrary units. Cr_u, urinary creatinine.

Urinary GM2AP coincides with renal cortical damage and grades cisplatin nephrotoxicity severity in rats

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Abbreviations
AKI, acute kidney injury.
BMI, body mass index.
CDDP, cisplatin.
Cl_{Cr}, clearance of creatinine.
Cr_p, plasma creatinine concentration.
Cr_u, urinary creatinine concentration.
DAPI, 4'6-diamidino-2-phenylindole.
ERBP, European Renal Best Practice.
FE_{Glu}, fractional excretion of glucose.
G, gentamicin.
Glu, glucose.
Glu_p, plasma glucose concentration.
Glu_u, urinary glucose concentration.
GFR, glomerular filtration rate.
GM2AP, ganglioside M2 activator protein.
IGFBP7, insulin-like growth factor binding protein 7.
IL-18, interleukin 18.
I/R, ischemia/reperfusion.
KDIGO, Kidney Disease: Improving Global Outcomes
KIM-1, kidney injury molecule 1.
LFABP, liver fatty acid binding protein.
NGAL, neutrophil gelatinase-associated lipocalin.
PSS, physiological saline solution.
TIMP-2, tissue inhibitor of metalloproteinase 2.
UF, urinary flow.

Abstract

Nephrotoxicity, including electrolytic disorders and acute kidney injury (AKI), limits the clinical dosage and utility of platinated antineoplastics such as cisplatin. Cisplatin nephrotoxicity embodies a tubulopathy involving the medullary S2 and S3 segments of the proximal and the distal tubules. Higher dosage extends damage over the cortical S1 segment and intensifies overall injury. However, the standard diagnosis based on plasma creatinine as well as novel injury biomarkers lacks enough pathophysiological specificity. Further granularity in the detection of renal injury would help understand the implications of individual damage patterns needed for personalized patient handling. In this article, we studied the association of urinary ganglioside GM2 activator protein (GM2AP) with the patterns of tubular damage produced by 5 and 10 mg/kg cisplatin in rats. Our results show that GM2AP appears in the urine only following damage to the cortical segment of the proximal tubule. The information provided by GM2AP is not redundant with but distinct and complementary to that provided by urinary neutrophil gelatinase-associated lipocalin (NGAL). Similarly, treatment with 150 mg/kg/day gentamicin damages the renal cortex and increases GM2AP urinary excretion; whereas renal ischemia, which does not affect the cortex, has no effect on GM2AP. Because of the key role of the cortical proximal tubule in renal function, we contend GM2AP as a potential diagnostic biomarker to stratify AKI patients according to the underlying damage and follow their evolution and prognosis. Prospectively, urinary GM2AP may help grade the severity of platinated antineoplastic nephrotoxicity by forming part of a non-invasive liquid biopsy.

Key words: cisplatin, GM2AP, acute kidney injury, acute tubular injury, cortical necrosis, urinary biomarker

1. Introduction

Cisplatin is a widely used platinated chemotherapeutic drug, whose dosage and clinical utility are limited by significant side effects, mainly nephrotoxicity (Sanchez-Gonzlez et al. 2011) ranging from electrolytic disorders to acute kidney injury (AKI) (Blachley and Hill 1981). AKI is an ambiguous term for a multi-etiological and heterogeneous syndrome characterized by a sudden decrease in glomerular filtration rate (GFR) (Basile et al. 2012), with high incidence and derived morbimortality in the short and long term (Doyle and Forni 2016). AKI is especially disastrous for critically ill patients, among whom incidence and mortality escalate to excruciating rates of up to 50% (Ronco et al. 2019). From a pathological perspective, AKI has been traditionally sub classified in pre-renal (i.e., mostly hemodynamic, with no renal parenchymal damage), intrinsic (i.e., derived from primary renal tissue injury, most typically involving the tubule epithelium), and post renal forms (i.e., resulting from obliteration of the urinary ways) (Ronco et al. 2019).

Specifically, cisplatin nephrotoxicity encompasses a tubulopathy affecting the proximal and distal tubules, where the drug mostly accumulates (Sanchez-Gonzlez et al. 2011; Sancho-Martínez et al. 2012). Within the proximal tubule, cisplatin preferentially accumulates and damages the S3 straight segment (Dobyan et al. 1980; Fenoglio et al. 2002; Price et al. 2004; Cristofori et al. 2007; Perše and Večerić-Haler 2018), located mainly in the outer strip of the outer medulla (Zalups et al. 2014). As dosage is increased, the cortical, convoluted S1 and S2 segments also become injured (Jones et al. 1985) resulting in different damage patterns with potentially distinct impact, evolution, and prognosis. Accordingly, precise pathophysiological diagnosis based on the underlying damage pattern is necessary (Moledina and Parikh 2018), for which present diagnostic technology lacks optimal performance. Internationally recognized criteria for AKI diagnosis (the latest of which being the KDIGO and ERBP criteria) rely on increments in plasma creatinine concentration (Cr_p), or on a reduction in urinary output (Fliser et al. 2012; Thomas et al. 2015). Cr_p is an inverse surrogate of GFR with several important limitations, including low sensibility and no specificity on the cause, type, or injury phenotype of AKI (Makris and Spanou 2016). In fact, Cr_p may increase during AKI regardless of the level, and even in the absence of tubular damage (as in pre-renal AKI).

Consequently, Cr_p-blind damage needs to be profiled with new diagnostic methods. In this sense, a generation of so-called *injury biomarkers* (mostly urine-borne) is gaining increasing attention and slowly erupting into the clinical scenario, for their association with renal injury independently, and even in the absence, of increments in Cr_p. These biomarkers most typically include kidney injury molecule 1 (KIM-1), neutrophil gelatinase-associated lipocalin (NGAL), interleukin 18 (IL-18), liver fatty acid binding protein (LFABP), tissue inhibitor of metalloproteinase 2 (TIMP-2) and insulin-like growth factor binding protein 7 (IGFBP7) (Vanmassenhove et al. 2019). However, the definition, classification, and diagnosis of subclinical AKI by means of "injury biomarkers" is still a quite vague concept. Patients positive for a specific biomarker may be, in turn, negative for another. This suggests that each biomarker has a particular biological and diagnostic meaning arising from a yet undetermined but distinctive damage pattern or specific pathophysiological process. Thus, further investigation and knowledge on their etiology, origin, and traffic among biological fluids and compartments is needed to endow these biomarkers with net clinical significance and utility.

With this aim, this article focuses on ganglioside GM2 activator protein (GM2AP), a novel and sensitive injury biomarker candidate whose urinary level has been previously related to AKI. GM2AP increases in the urine of different animal models of toxic AKI (Sancho-Martínez et al. 2022). Urinary GM2AP shows high sensitivity and early diagnostic capacity. Indeed, GM2AP appears in the urine as a consequence of defective tubular reabsorption, even under subclinical AKI conditions (i.e., normal Cr_p), and largely precedes Cr_p elevation during overt AKI (Quiros et al. 2010). GM2AP also increases in the urine of patients undergoing AKI in different clinical contexts (Blanco-Gozalo et al. 2020; Vicente-Vicente et al. 2021), and contributes to anticipating delayed recovery and bad prognosis (Blanco-Gozalo et al. 2020).

In this article, we provide further pathophysiological contextualization of urinary GM2AP as a marker of renal cortical damage allowing for gradation of cisplatin nephrotoxicity severity.

2. Materials and Methods

Where not otherwise indicated, reagents were purchased from Sigma-Aldrich.

ARRIVE Statement

Animal experiments were carried out in compliance of ARRIVE Guidelines.

2.1. Animals and experimental protocol

All procedures were approved by the Bioethics Committee of the University of Salamanca and the Regional Government of Castile and Leon, Ministry of Agriculture and Livestock. Animals were handled according to the guidelines of the European Community Council Directive 2010/63/UE, and to the current Spanish legislation for experimental animal use and care (RD 53/2013). Male Wistar rats (220−240 g; Charles River, Madrid, Spain) were used and maintained under controlled environmental conditions within the University of Salamanca Animal House facility, with free access to water and standard chow. Four types of experiments were performed (Figure 1):

- Experiment 1: rats were divided into the following groups (n=3) per group: (i) Control: rats received physiological saline solution (PSS, the vehicle) i.p.; (ii) Cisplatin-5 (CDDP5): a single i.p. injection of 5 mg/kg cisplatin; (iii) Cisplatin-10 (CDDP10): a single i.p. injection of 10 mg/kg cisplatin. Animals were followed for 4 days. In these rats, renal function (section 2.2.) and renal histology (section 2.3.) were assessed as described below. For these purposes, rats were allocated in metabolic cages at specific time points for individual urine collection. Urine was cleared by centrifugation and stored at -80 ºC. Blood was obtained from a small incision in the tail vein and immediately centrifuged to obtain the plasma, which was kept at -80 ºC until use. At day 4, rats were anesthetized with 80 mg/kg ketamine + 20 mg/kg xylazine and their kidneys perfused with saline solution to remove the blood, dissected and immediately fixed in 3,7% p-formaldehyde for histological studies.
- Experiment 2: in a separate set of experiments, rats (n=3 per group) were treated as above and, 4 days after cisplatin treatment, *in situ* renal cell viability studies were performed as described below (section 2.4.).
- Experiment 3: in a third set of experiments, rats (n=3 per group) were treated as above and, 4 days after cisplatin treatment, were anesthetized (as above) and the jugular vein and the urinary bladder were cannulated. A single injection of sodium maleate (400 mg/kg), a proximal tubule reabsorption inhibitor (Christensen and Maunsbach 1980;

Cho et al. 1985; Hysting et al. 1987; Uwai and Nabekura 2021), was administered through the jugular catheter. Urine was collected from the bladder catheter during 30 minutes before maleate administration, and for 90 minutes thereafter, centrifuged, and kept at -80 ºC.

- Experiment 4: in a fourth set of experiments, rats (n=3 per group) were divided into the following groups: (i) Control: rats received physiological saline solution (PSS, the vehicle) i.p.; (ii) Gentamicin (G): rats received 6 daily, consecutive i.p. injections of 150 mg/kg gentamicin; (iii) Ischemia (I/R): Renal ischemia/reperfusion was carried out basically as described (Paniagua-Sancho et al. 2021). Briefly, rats were anesthetized with a mixture of 80 mg/kg ketamine and 20 mg/kg xylazine. Kidneys were accessed through a medial laparotomy and the left renal artery gently exposed, cleaned from surrounding fat, and clamped for 60 minutes, after which the clamp was removed to allow for reperfusion. The right hilum was ligated, and the kidney was dissected. Incisions were then sutured, and animals were allowed to recover under 30 μg/kg buprenorphine analgesia. 24 hours after the ischemic procedure (in the I/R group) and 7 days after inception of the gentamicin regime (in the G group), blood samples, and renal tissue specimens were obtained, processed, and kept as described above. Cr_p was measured (as indicated in section 2.2.), and histological assessment was performed on renal specimens as described in section 2.3.

In all cases, GM2AP was measured in the urine as described in section 2.5.

2.2. Renal function analysis

GFR was monitored by means of Cr_p and creatinine clearance (Cl_{Cr}). Cl_{Cr} was calculated using the equation $Cl_{Cr} = (Cr_u \times UF)/ Cr_p$, where Cr_u stands for the urinary concentration of creatinine, and UF for urinary flow. Cr_p and Cr_u were measured with a commercial, colorimetric assay based on the Jaffe reaction (*Quantichrom Creatinine Assay Kit*, BioAssay Systems, Hayward, CA, USA). Tubular performance was assessed by means of the fractional excretion of glucose (FE_{Glu}), defined as $(Glu_u \times Cr_p) / (Glu_p \times Cr_u)$, where Glu_u and Glu_p stand for the concentration of glucose in the urine and plasma, respectively. Glu_u and Glu_p were measured with commercial reactive strips (Bayer, Leverkusen, Germany).

2.3. Renal tissue assessment

For histopathological studies, paraffin blocks were made with 3.7% p-formaldehyde-fixed kidneys, and 5-μm tissue sections were stained with hematoxylin and eosin for visual inspection under the microscope. Renal injury was blindly determined by means of a "severity score", which was calculated semi quantitatively as described (14). A score of 0–3 was assigned to each section, according to the following criteria: 0, normal histology; 1, tubular cell swelling, brush border loss, nuclear condensation, or loss of nuclei in up to one third of the tubular perimeter; 2, same as for 1, but from one third to two third of nuclei loss; and 3, over one third of nuclei loss. Section scores were added to give a field score (maximal score per field = 30). The average score of 10 fields was used for each kidney sample.

2.4. *In situ* evaluation of renal cell viability with ethidium homodimer

Ethidium homodimer is a cell impermeable, DNA-binding, fluorescent dye excluded from viable cells, used for *in situ* cell viability studies (Edwards et al. 2007, 2020; Prozialeck et al. 2009). For this purpose, on day 4, animals were anesthetized with 80 mg/kg ketamine + 20 mg/kg xylazine. A laparotomy was performed to expose the left

kidney, which was perfused through a catheter placed in the aorta after ligation of the right renal and main mesenteric arteries, and the aorta right above and below the catheter. Perfusion was carried out with 5 µM ethidium homodimer in PSS for 5 minutes at a 1 mL/min flow, then for another 5 minutes at 3 mL/min and, finally, with PSS at 3 mL/min for 10 minutes (to eliminate the excess ethidium homodimer). Perfusion pressure was monitored during the whole process with a pressure transducer connected to a MP150 digital data recorder (Biopac Systems, Goleta, CA, USA), and verified to not exceed 100 mmHg. After perfusion, the left kidney was dissected, and cut longitudinally into two halves. One was fixed in 3,7% paraformaldehyde and used for histological studies (as described in the next section), and the other half was preserved in 30% saccharose in PBS (138 mM NaCl, 2.6 mM H_2KPO_4, 4.1 mM Na_2HPO_4) for fluorescence visualization. These specimens were fixed with ice-cold methanol, embedded in paraffin, cut into 5 µm slices, placed on glass slides, stained with 1 mg/mL 4'6-diamidino-2-phenylindole (DAPI), preserved with DPX mounting medium under coverslips and visualized and photographed under an Axiovert 200M inverted microscope (Zeiss, Madrid, Spain). To quantify cortical necrosis, photographs of 10 cortical fields per specimen were evaluated. Cortical necrosis was evaluated as the average % of ethidium homodimer-positive cells in the 10 fields, out of all cells (i.e., ethidium homodimer-positive + DAPI-positive cells).

2.5. Urinary GM2AP measurement

Western blot was used to measure urinary GM2AP. Western blot was run with a volume of urine from each animal corresponding to the same fraction of urinary output. Urine samples were separated by acrylamide electrophoresis. Proteins were transferred to an Immobilon-P Transfer Membrane (Millipore, Madrid, Spain) and incubated with anti-GM2AP polyclonal antibody produced for our laboratory (by Immunostep, Salamanca, Spain) as described (10), followed by a horseradish peroxidase-conjugated goat anti-rabbit secondary antibody (Southern Biotech, Birmingham, AL, USA). A positive control was used in all experiments to normalize biomarker levels, and all biomarkers were expressed as arbitrary units (% of positive control, which was assigned the 100% value). Bands were quantified and normalized to the intensity of the positive control. The positive control was urine from a highly proteinuric rat urine obtained and tested positive for GM2AP in pilot studies.

2.6. Patients and clinical protocol

An observational study, designed as proof-of-concept, was conducted to pre-emptively evaluate the change in GM2AP urinary level in a clinical scenario with cancer patients from the Medical Oncology Service of the University Hospital of Salamanca (Salamanca, Spain) following treatment with the antineoplastic drug cisplatin. The study fulfilled the principles established in the accordance with the Declaration of Helsinki (World Medical Assembly), the Council of Europe Convention on Human Rights and Biomedicine, the UNESCO Universal Declaration on the Human Genome and Human Rights, the requirements established in the Spanish legislation in the field of biomedical research, personal data protection and bioethics; as well as the provisions of the Law 14/2007, of July 3rd, of Biomedical Research; and RD 53/2013, of February1st. The study was approved by the University Hospital of Salamanca Ethical Committee and all patients provided written consent. The following information was collected from each patient: sex, age, weight, height, body mass index (BMI), body surface area and cisplatin dose. Urine and blood samples were collected immediately before and 72 hours after cisplatin administration. The following analytical data were extracted from their medical records before and after the first chemotherapy cycle: plasma creatinine, plasma urea, calcemia and magnesemia. GM2AP and NGAL were measured in urine samples. GM2AP was

determined as described above, but in this case 21 µL urine from each patient were used. NGAL was measured with a commercial ELISA (Human NGAL ELISA Kit 036CE, BioPorto Diagnostics, Hellerup, Denmark) following the manufacturer's instructions. Both GM2AP and NGAL data were normalized by the corresponding urinary creatinine concentration (Cr_u) in each sample.

2.7. Statistical analysis

Data normality was evaluated with the Shapiro–Wilk test. For data with a normal distribution, two-way ANOVA was applied and the Dunnett´s or Tukey´s post hoc tests were used for multiple and simple comparisons. For non-normal data, Kruskal-Wallis and Dunn´s tests were used. On the other hand, Pearson's correlation tests (for normal data) or Spearman's (for non-normal data) were applied to evaluate the association among patient data parameters. In all cases, statistical significance was established at $p < 0.05$. Statistical analysis was performed with the GraphPad Prism 7.0 software (GraphPad Software, San Diego, CA, USA).

3. Results

3.1. Increasing cisplatin dosages produce increasing cortical tubular necrosis despite causing a similar level of AKI

We (and others) have previously showed that 5 mg/kg cisplatin causes marked tubular injury restricted to the outer strip of the outer renal medulla (Casanova et al. 2020, 2021; Sancho-Martínez et al. 2020; Fuentes-Calvo et al. 2021; Cuesta et al. 2022a). In pilot studies we realized that a higher dose of cisplatin (10 mg/kg) caused renal parenchymal damage extending to the renal cortex in rats. Collectively, Figures 2, 3 and 4 show that despite causing an almost identical level of AKI, as measured by the international criterium (i.e., plasma creatinine concentration), and almost identical renal medullary affection, 10 mg/kg cisplatin caused a marked cortical necrosis that is absent following administration of 5 mg/kg cisplatin. Figure 2a shows that both dosages of cisplatin cause a similar increment in the gold standard AKI diagnostic parameter, Cr_p, and a similar reduction in GFR measured as clearance of creatinine. However, more extensive proximal tubular affection is evident after 10 mg/kg than after 5 mg/kg cisplatin from the higher fractional excretion of glucose (Figure 2b), and the cortical damage observed in the histological study. Figure 3 depicts representative images of both the cortical and the outer medullary areas of rats treated with no (controls), 5 and 10 mg/kg cisplatin. For a similar medullary involvement, 10 mg/kg cisplatin produces marked cortical damage characterized by extensive tubular necrosis, which is not observed in the other groups. *In vivo* labelling of necrotic cells with ethidium homodimer shows very similar results (Figure 4). Whereas substantial necrosis is detected after 10 mg/kg cisplatin in the cortical area, only a low level is seen after 5 mg/kg.

3.2. GM2AP appears in the urine coinciding with cortical injury and after inhibition of proximal tubule reabsorption

Interestingly, the tubular injury marker GM2AP is elevated in the urine only when rats are treated with 10 mg/kg and not (or minimally) with 5 mg/kg cisplatin, compared with controls (Figure 5a). As a corollary, the AKI group bearing cortical damage excretes GM2AP in the urine, whereas the AKI group not showing cortical involvement does not. In addition, inhibition of proximal tubule transport with sodium maleate in normal rats also caused an increase in the urinary excretion of GM2AP (Figure 5b), showing a potential mechanism explaining GM2AP urinary excretion in rats with damaged proximal

tubules. Moreover, an additional insult causing renal cortical damage (i.e., the aminoglycoside antibiotic gentamicin) also shows increased urinary excretion of GM2AP; whereas I/R, which produces no harm to the renal cortex, does not (Figures 6 and 7).

3.3. GM2AP in patients treated with cisplatin

With an aim to gaining a first, pilot view on whether GM2AP is also excreted in the urine of cisplatin-treated patients, we studied a group of cancer patients to be treated with this drug who did not excrete GM2AP prior to the oncological therapy (or had only minimal excretion) and who were otherwise unselected according to any other characteristics. If GM2AP behaved in humans according to the experimental model, patients might show increased or unaltered GM2AP urinary excretion following cisplatin administration, depending on the degree of proximal tubule affection. Patient characteristics are depicted in Table 1. As shown in Figure 8, urinary GM2AP was not increased by cisplatin therapy in patients # 2, 6, 8 and 10, whereas it was elevated in patients 1, 3, 4, 5, 7, 9 and 11 to variable degrees after cisplatin administration. The pathophysiological (and potential diagnostic) information provided by urinary GM2AP is not redundant but complementary to that drawn from another tubular injury biomarker, i.e., NGAL, which had a distinctive urinary excretion pattern. In fact, the incremental excretion of GM2AP (i.e., the difference between the excretion prior to cisplatin and that observed after cisplatin) showed no correlation with any other parameter related to the impact of cisplatin on renal function and integrity (Table 2).

4. Discussion

Two critical elements of a prospective pathophysiological diagnosis are the type of damage and the tubular segments involved. To this end, this article provides new information on the meaning of urinary GM2AP on proximal tubule pathophysiology. The proximal tubule is a highly susceptible part of the nephron and, by reabsorbing the majority of the ultrafiltrate, a determinant of overall nephron function. Proximal S1, S2, and S3 segments have distinct anatomical, histological, biochemical, and functional characteristics, and differential susceptibility to injury and to specific insults (Venkatachalam et al. 1978; Christensen et al. 2021). In fact, the S3 has been proposed to provide functional reservoir for S1 and S2. When reabsorption in S1 and S2 is overwhelmed, the S3 is recruited (Christensen et al. 2021). However, the impact of damage to each proximal tubule segment on disease progression and outcome remains unknown due to the absence of sufficiently granular diagnosis. Our results show that the increased urinary excretion of GM2AP occurs in coincidence with the dosages of cisplatin that produce cortical tubular damage and thus unveil GM2AP as a potential candidate biomarker of damage to the S1 and S2 segments.

The dose-dependent cortical damage induced by cisplatin is recapitulated in our experimental model. It is not until the S1 and S2 segments become damaged that GM2AP urinary excretion is increased. In agreement, urinary GM2AP was previously shown to increase also in a rat model of gentamicin nephrotoxicity (Quiros et al. 2010), a drug known to affect the S1 and S2 segments of the proximal tubule (Wedeen et al. 1983; Zhang et al. 2009; Lopez-Novoa et al. 2011). GM2AP seems to detect a wide range of S1/S2 damage levels, ranging from subtle alterations in the reabsorption machinery (such as in gentamicin-induced predisposition to AKI or following exposure to maleate (Quiros et al. 2010)), to overt S1/S2 necrosis (i.e., as induced by gentamicin or high dose

cisplatin), in which tubules are incapable of normally reabsorbing the filtered GM2AP (Quiros et al. 2010).

Identification of the type of damage (i.e., from sublethal, functional alterations to tubular necrosis) is of critical diagnostic interest since unrepaired damage has direct impact on outcome. Specifically, AKI involving parenchymal damage associates with worse evolution and prognosis (Liaño et al. 1996; Esson and Schrier 2002; Bellomo et al. 2012; Uchino et al. 2012; Rachoin et al. 2012; Endre et al. 2013; Yang et al. 2014; Sawhney et al. 2015; Barasch et al. 2017). In fact, damaged tubules have a remarkable but limited capacity of repair. Following an AKI, proximal tubules regenerate but only to a shorter length, with repeated AKI episodes accumulating further reductions (Endo et al. 2015). Together with irreversible nephron loss (Kellum et al. 2021) and persisting tubular atrophy with dedifferentiated tubule cells stagnated in a profibrotic phenotype (Bonventre 2014; Venkatachalam et al. 2015), tubule shortening contributes to maladaptive repair and progression to chronic kidney disease (Ferenbach and Bonventre 2015; Venkatachalam et al. 2015). In fact, repeated AKI has been shown to independently provide cumulative risk for developing CKD (Thakar et al. 2011). In the short term, sub-clinical renal tissue sequelae (i.e., persisting after Cr_p normalization) (Fuentes-Calvo et al. 2021; Cuesta et al. 2022b) correlate with abnormally increased and remaining susceptibility to new AKI episodes (Fuentes-Calvo et al. 2021).

Because urinary GM2AP provides segment specificity but not information on the type of damage (i.e., potentially ranging from sublethal, functional alteration to overt tubular necrosis), complementary biomarkers are needed to contextualize its precise meaning in each AKI case. *Injury biomarkers* pose immediately obvious candidates. However, the pathophysiological meaning of most (if not all) *injury biomarkers* is still incompletely understood. For example, NGAL, TIMP-2 and IGFBP7 were long regarded as markers of tubular injury directly shed to the urine by damaged tubules. Nevertheless, recent studies (Johnson and Zager 2018; Sancho-Martínez et al. 2020; Skrypnyk et al. 2020) have shown that their increased urinary excretion following acute renal injury is mostly (and probably only) due to defective tubular reabsorption. These findings extend their diagnostic sensitivity to milder (i.e., sub-lethal) and even subclinical forms of damage, but reduce their specificity. An increased urinary excretion of these injury biomarkers may be as reflective of subtle alterations in tubular function as of overt tubular necrosis. This is further complicated by the ambiguous relation of the extent of structural tubular damage and the urinary level of injury biomarkers (Sancho-Martínez et al. 2022), an effect that may be partly explained by sub-lethal alterations impacting biomarker reclamation. Consequently, an unmet diagnostic need and a challenge for the future is to identify urine-borne, segment-specific biomarkers of tubule cell death and tissue derangement.

Our results also support that, rather than redundant, injury biomarkers seem to be mostly complementary. In fact, the excretion pattern of GM2AP in the unselected group of patients included in this study has no similitude with that of NGAL. Injury biomarkers do not function in unison and, thus, cannot be used collectively as a class, as frequently proposed. In fact, a redefinition of AKI including injury biomarkers is increasingly suggested, which proposes three diagnostic phenotypes as per increments in either Cr_p or injury biomarkers, or in both (Moledina and Parikh 2018; Desanti De Oliveira et al. 2019; Ronco et al. 2019; Ostermann et al. 2020). Our results support that, on the contrary, injury biomarkers must be used according to their specific biological meaning as parts of additive fingerprints.

GM2AP may be proposed as a candidate marker to monitor the extent of tubular damage and the severity of cisplatin nephrotoxicity and, also, of subclinical nephrotoxicity, as its urinary levels increase in specific patients in whom no increments in Cr_p are evidenced. Indeed, tubular alterations occurring with normal Cr_p, are typical of therapeutic courses with platinated antineoplastics, resulting in hypomagnesemia and hypocalcemia (Casanova et al. 2020). Interestingly, GM2AP contributes supplemental pathological information on tubular injury, which is blind to these electrolytic disturbances, as low and insignificant correlations exist between them.

Moreover, GM2AP might provide additional diagnostic utilities. For instance, the relation between cisplatin dose and injury extension seen in animal models may be partially distorted or uncoupled in patients by concomitant therapies frequently used in combined anticancer therapies. In these circumstances, a liquid biopsy (potentially including GM2AP) may better profile the actual renal damage inflicted by the specific treatment in each individual patient, for a personalized handling with precision medicine criteria. Regulated and structured clinical studies involving appropriate cohort sizes are now necessary to deeply understand the incremental diagnostic value of urinary GM2AP on the limitations of traditional clinical parameters.

5. Conclusions

In summary, or study unveils urinary GM2AP as a candidate biomarker of damage to the renal proximal tubule S1 and S2 segments with subclinical sensitivity, contributing increased granularity to a prospective pathophysiological diagnosis of AKI. Furthermore, in the case of treatments with platinated antineoplastics such as cisplatin, the presence of GM2AP in the urine may be indicative of more severe parenchymal damage extending to the cortical area.

6. Acknowledgements.

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Table 1. Patient characteristics. BMI, body-mass index. ND, not determined. Post, 72 h after cisplatin. Pre, before cisplatin administration.

Patient	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**
Sex	Male	Male	Female	Male	Male	Female	Male	Male	Male	Female	Male
Age (years)	45	53	68	54	62	41	43	60	64	53	52
Weight (kg)	103	81	70	84	71	61	90	60	65	68	75
Height (cm)	177	165	148	170	165	148	181	173	163	159	175
BMI (kg/m^2)	32.9	29.8	32.0	29.1	25.9	27.8	27.5	20.0	24.5	26.9	24.5
Body Surface (m^2)	2.2	1.9	1.6	1.9	1.8	1.5	2.0	1.8	1.7	1.8	1.9
Cisplatin dose (mg)	150	110	80	47	140	110	150	135	120	100	135
Plasma creatinine Pre/Post (mg/dL)	0.95/ 1.04	0.72/ND	0.70/ND	1.16/ 1.17	0.65/ND	0.72/ 0.66	1.34/ 0.76	0.98/ 1.11	0.80/ 0.71	0.71/ND	0.74/ 0.76
Plasma urea Pre/Post (mg/dL)	40/ 50	35/ND	32/ND	49/ 49	40/ND	20/ 19	71/ 42	38/ 56	30/ 22	25/ND	37/ 35
Calcemia Pre/Post (mg/dL)	8.8/ 8.4	9.5/ND	9.8/ND	11.0/ 9.9	8.8/ND	8.9/ 8.4	9.1/ 8.3	9.7/ 7.5	9.5/ 8.8	9.5/ND	9.6/ 9.4
Magnesemia Pre/Post (mg/dL)	2.1/ 2.1	ND/ND	1.8/ND	1.5/ 1.8	2.3/ND	1.8/ 2.2	2.0/ 2.4	2.2/ 1.8	2.1/ 2.4	2.0/ND	1.9/ 2.0

Table 2. Correlation between cisplatin dose and biomarker level in patients. Data represent Pearson's R coefficient (p-value) for normal data or Spearman's rho coefficient (p-value) for non-normal data. Δ, increment from immediately before cisplatin administration to 72 hours afterwards. *, p<0.05; **, p<0.01.

	Cisplatin dose	Δ Plasma creatinine	Δ Plasma urea	Δ Calcemia	Δ Magnesemia	Δ NGAL	Δ GM2AP
Cisplatin dose		0.127 (0.786)	-0.110 (0.814)	0.115 (0.807)	-0.338 (0.458)	0.207 (0.556)	0.456 (0.159)
Δ Plasma creatinine			0.893 (0.007)**	0.000 (1.000)	-0.821 (0.023)*	0.250 (0.589)	0.643 (0.119)
Δ Plasma urea				-0.406 (0.365)	-0.779 (0.039)*	0.131 (0.780)	0.474 (0.282)
Δ Calcemia					0.633 (0.127)	-0.464 (0.295)	0.100 (0.831)
Δ Magnesemia						-0.611 (0.145)	-0.525 (0.227)
Δ NGAL							0.146 (0.687)
Δ GM2AP							

Figures

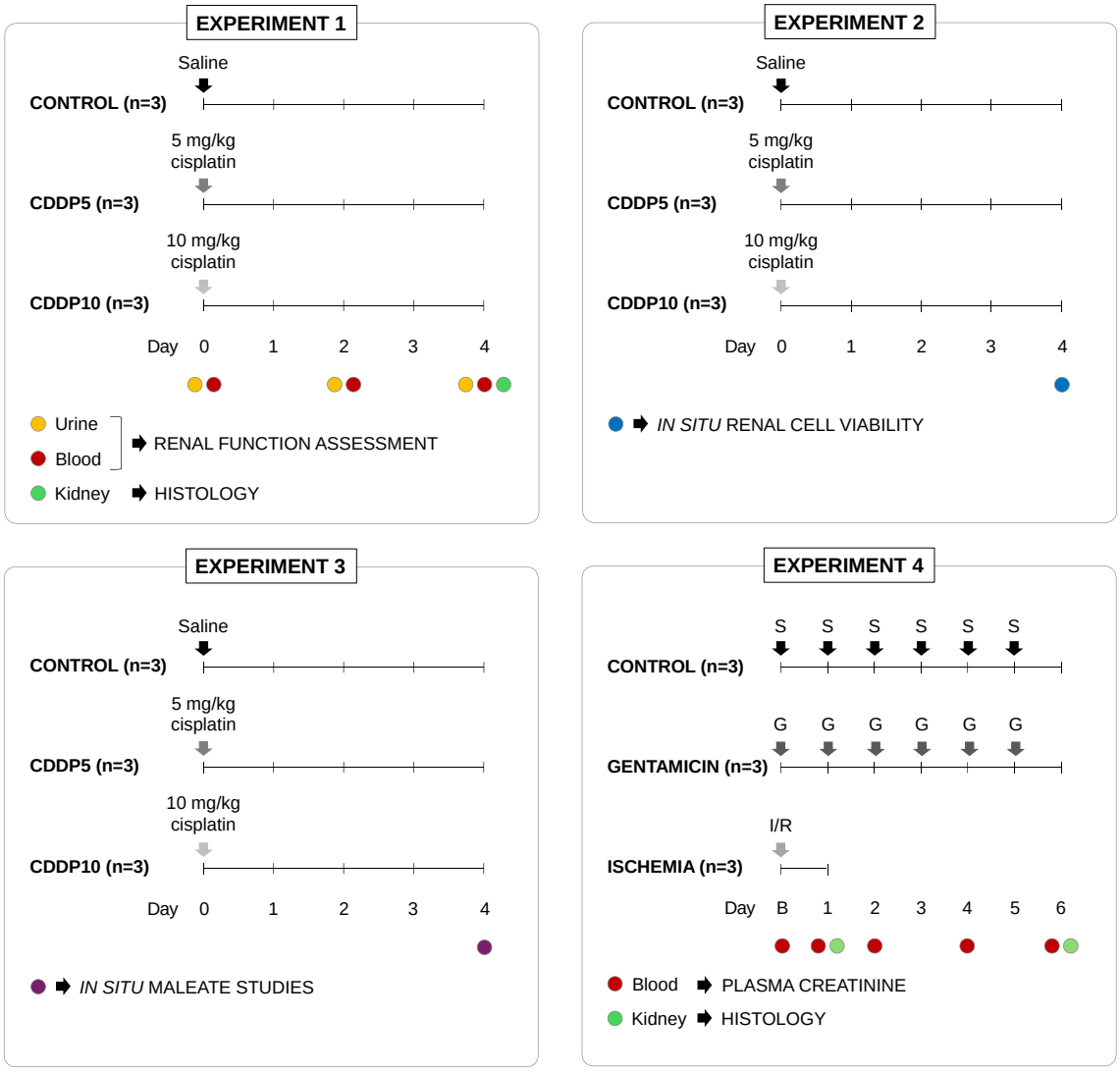


Figure 1. Schematic representation of the experimental protocols used in this study. B, basal. CDDP, cisplatin. G, gentamicin. I/R, ischemia/reperfusion. S, saline.

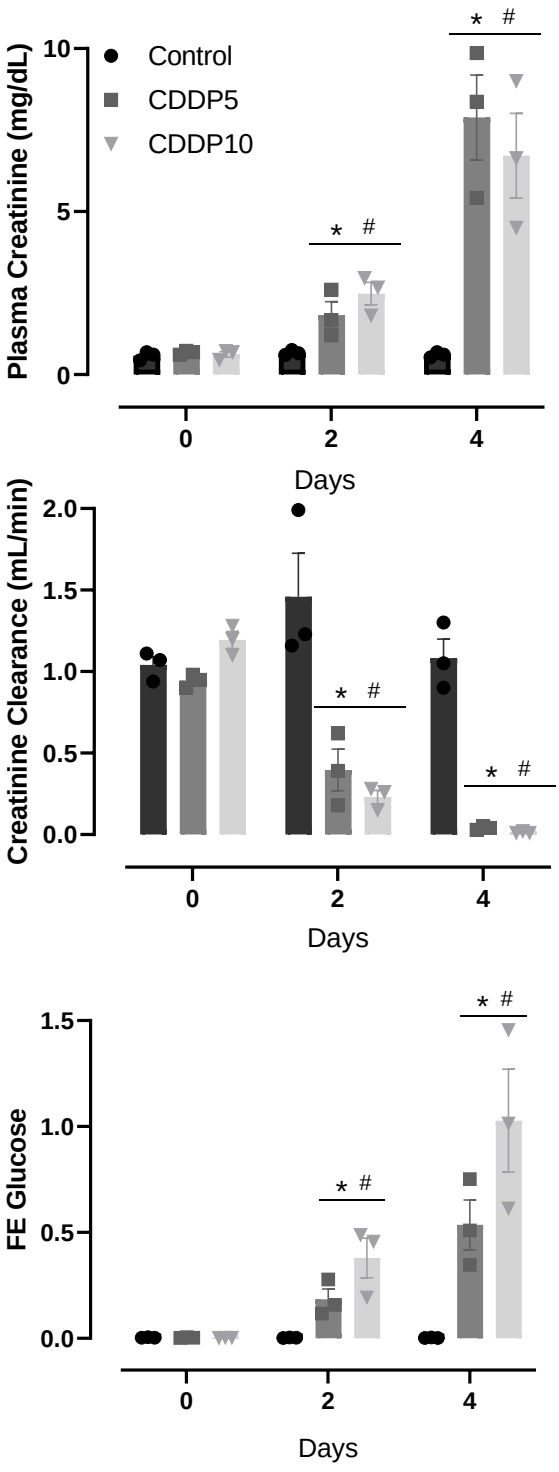


Figure 2. Renal function assessment following treatment with cisplatin. (a) Plasma creatinine concentration; **(b)** creatinine clearance; **(c)** fractional excretion (FE) of glucose, from rats treated with 5 or 10 mg/kg cisplatin. Data are expressed as the mean ± SEM of n=3 per group. *, $p<0.05$ vs the control (on the same day); and #, $p<0.05$ vs. day 0 in the same group. CDDP, cisplatin.

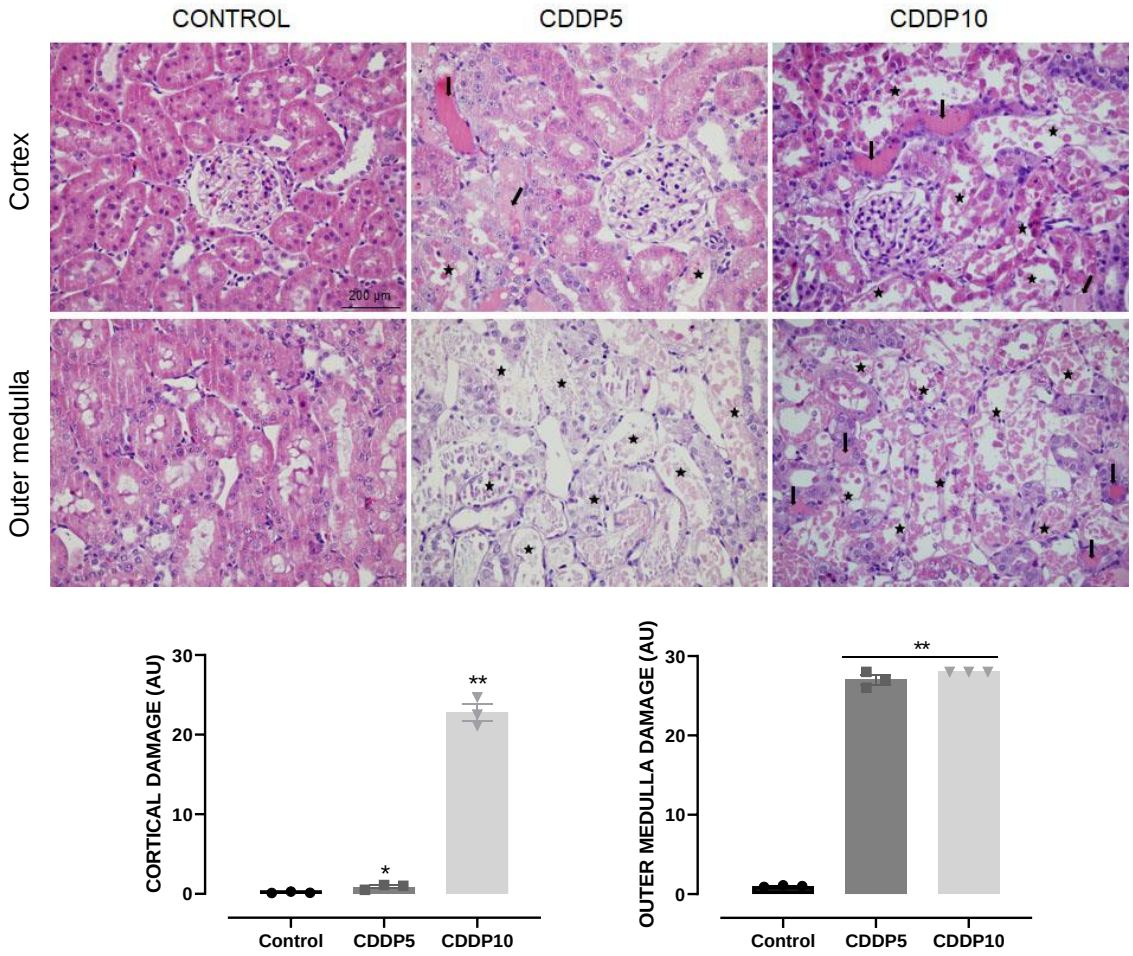


Figure 3. Renal tissue damage patterns produced by cisplatin. Upper panel show representative images (400x) of the cortical and outer medullary areas of renal sections from rats treated with 5 (CDDP5) or 10 mg/kg (CDDP10) cisplatin, stained with hematoxylin-eosin, showing tubular necrosis with tissue debris (*) and tubular obstruction (↓). The lower panels depict the corresponding damage scoring. Data are expressed as the mean ± SEM of n=3 per group. *, $p<0.05$ and **. $p<0.01$ vs the control. AU, arbitrary units.

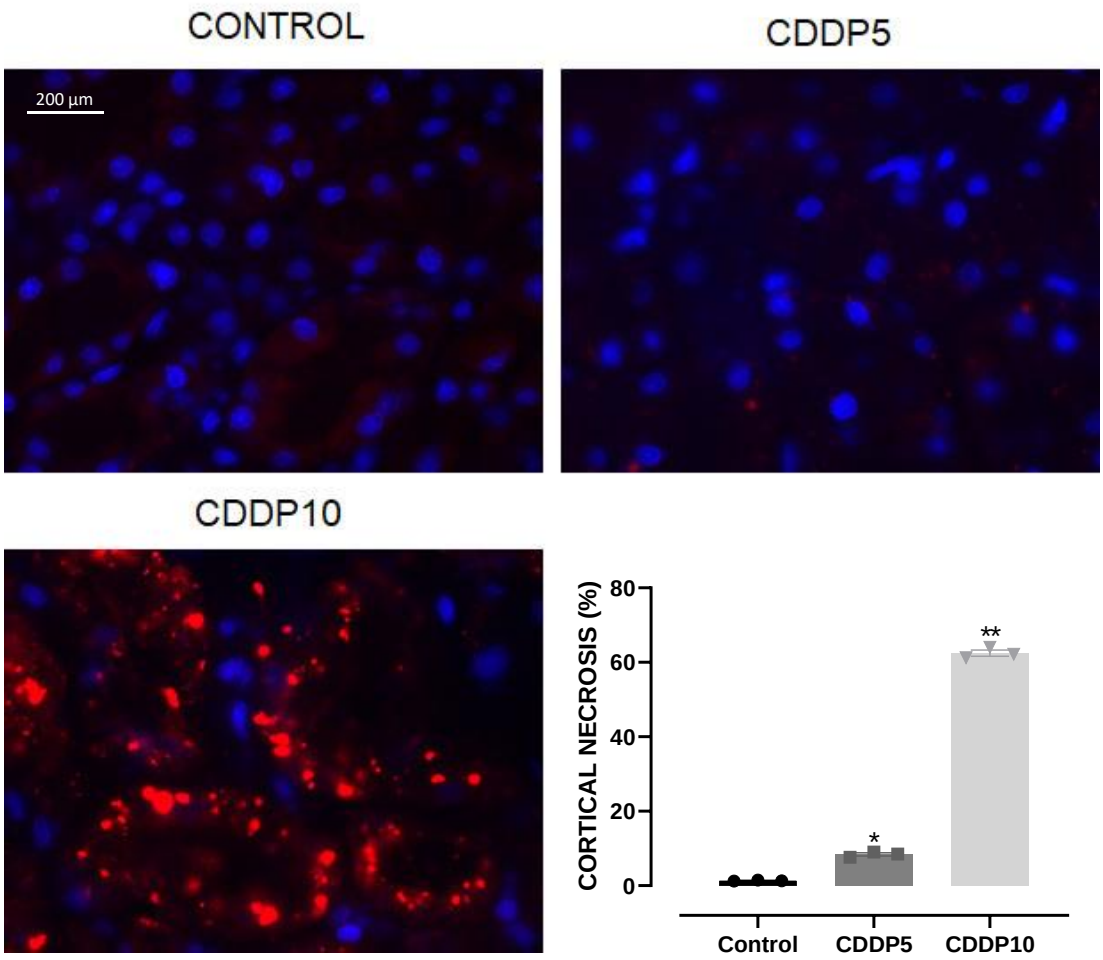


Figure 4. Cortical necrosis assessment following treatment with cisplatin. Representative images (400x) of the cortical area of renal sections from rats treated with 5 or 10 mg/kg cisplatin stained with ethidium homodimer, and the corresponding scoring (% of necrotic cells). Data are expressed as the mean ± SEM of n=3 per group. *, $p<0.05$. **, $p<0.01$ vs the control. CDDP, cisplatin.

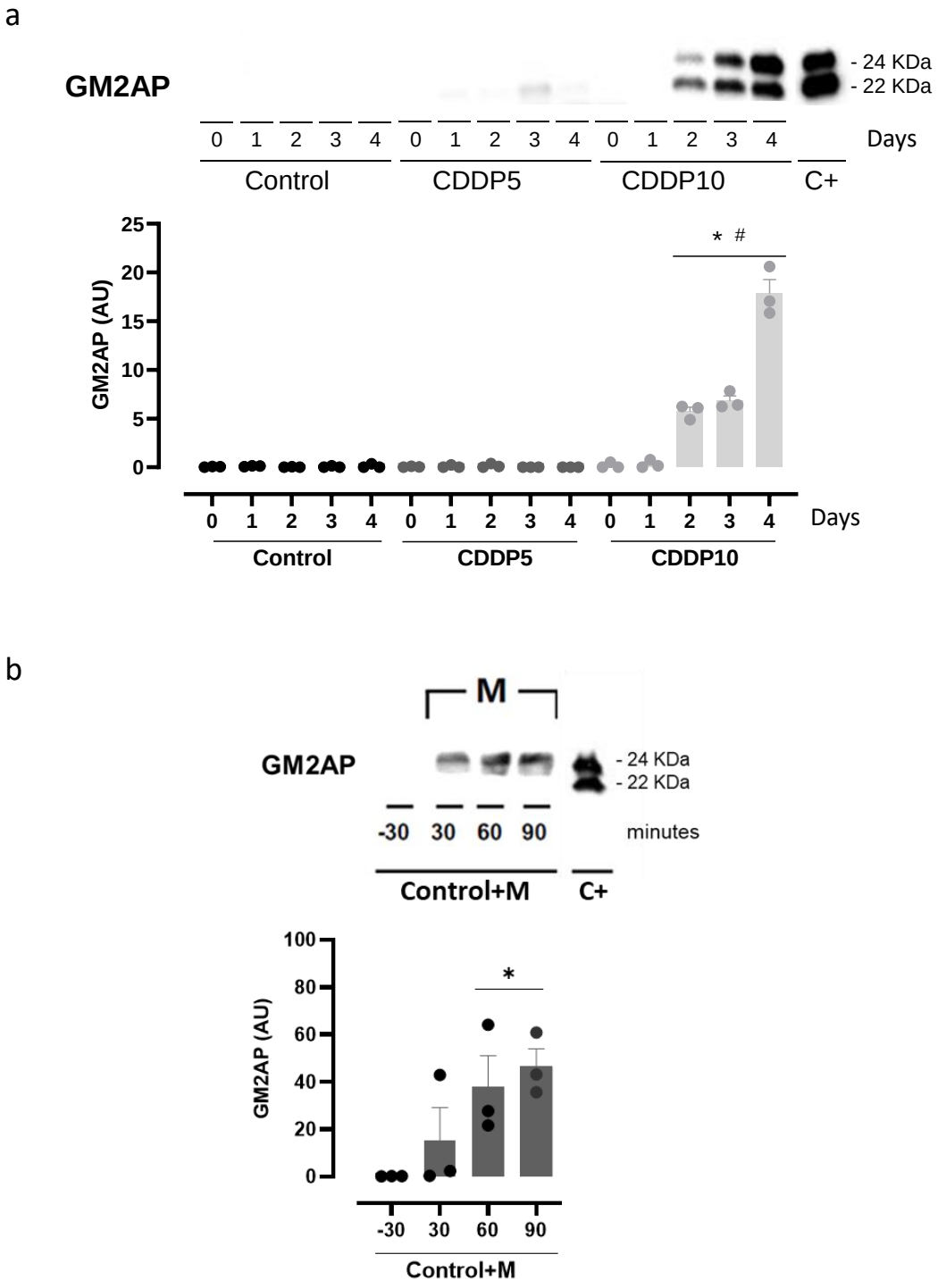


Figure 5. Urinary excretion of GM2AP caused by cisplatin. a) GM2AP appears in the urine following treatment with 10 but not with 5 mg/kg cisplatin. **b)** Effect of sodium maleate on GM2AP urinary excretion. Representative image of Western blot analysis for urinary GM2AP (upper panel), and the corresponding densitometric quantification (lower panel)**.** Data are expressed as the mean ± SEM of n=3 per group. *p<0.05 vs the control (on the same day); and #p<0.05 vs. day 0 in the same group. AU, arbitrary units. CDDP, cisplatin. C+, positive control. M, maleate.

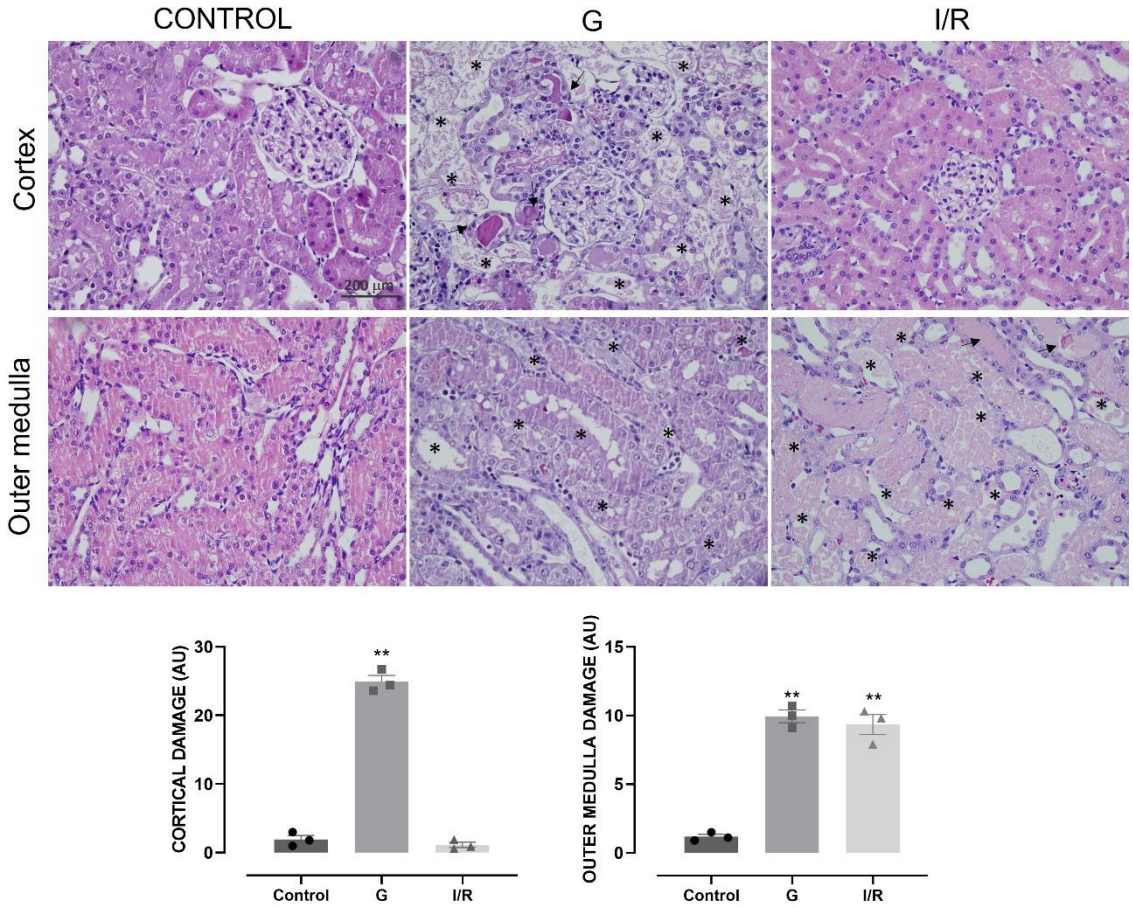


Figure 6. Renal tissue damage patterns produced by gentamicin and ischemia/reperfusion. Upper panel show representative images (400x) of the cortical and outer medullary areas of renal sections from rats treated with gentamicin (G) or subjected to renal ischemia/reperfusion (I/R), stained with hematoxylin-eosin, showing tubular necrosis with tissue debris (*) and tubular obstruction (↓). The lower panels depict the corresponding damage scoring. Data are expressed as the mean ± SEM of n=3 per group. **. $p<0.01$ vs the control. AU, arbitrary units.

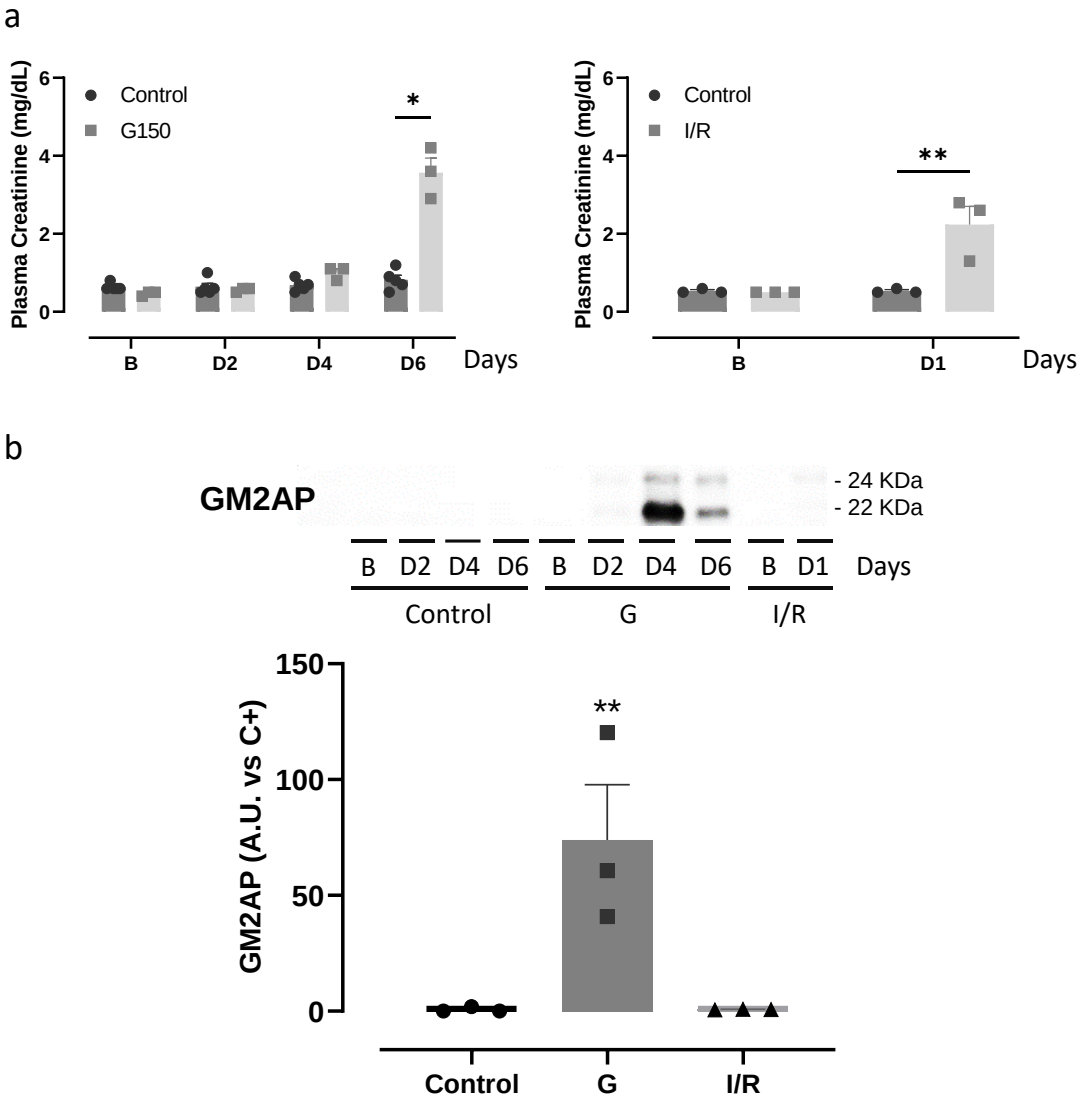


Figure 7. Renal function and urinary excretion of GM2AP following treatment with gentamicin and ischemia/reperfusion injury. (a) Plasma creatinine concentration; **(b)** urinary excretion of GM2AP of rats treated with gentamicin (G) or subjected to renal ischemia/reperfusion (I/R). Data are expressed as the mean ± SEM of n=3 per group. *, p<0.05, **. p<0.01, ***, p<0,001 vs the control. AU, arbitrary units.

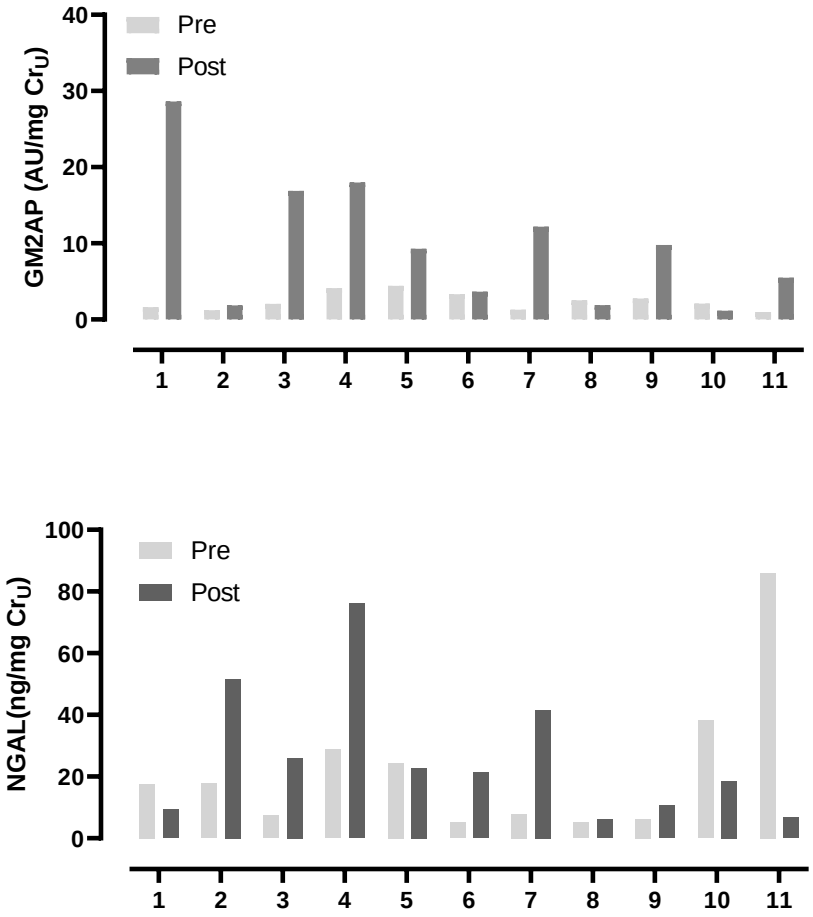


Figure 8. Profile of GM2AP and NGAL urinary excretion for 11 patients before (Pre) and 72 hours after (Post) cisplatin administration. AU, arbitrary units. Cr_u, urinary creatinine.

Table 1

Patient	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**
Sex	Male	Male	Female	Male	Male	Female	Male	Male	Male	Female	Male
Age (years)	45	53	68	54	62	41	43	60	64	53	52
Weight (kg)	103	81	70	84	71	61	90	60	65	68	75
Height (cm)	177	165	148	170	165	148	181	173	163	159	175
BMI (kg/m^2)	32.9	29.8	32.0	29.1	25.9	27.8	27.5	20.0	24.5	26.9	24.5
Body Surface (m^2)	2.2	1.9	1.6	1.9	1.8	1.5	2.0	1.8	1.7	1.8	1.9
Cisplatin dose (mg)	150	110	80	47	140	110	150	135	120	100	135
Plasma creatinine Pre/Post (mg/dL)	0.95/ 1.04	0.72/ND	0.70/ND	1.16/ 1.17	0.65/ND	0.72/ 0.66	1.34/ 0.76	0.98/ 1.11	0.80/ 0.71	0.71/ND	0.74/ 0.76
Plasma urea Pre/Post (mg/dL)	40/ 50	35/ND	32/ND	49/ 49	40/ND	20/ 19	71/ 42	38/ 56	30/ 22	25/ND	37/ 35
Calcemia Pre/Post (mg/dL)	8.8/ 8.4	9.5/ND	9.8/ND	11.0/ 9.9	8.8/ND	8.9/ 8.4	9.1/ 8.3	9.7/ 7.5	9.5/ 8.8	9.5/ND	9.6/ 9.4
Magnesemia Pre/Post (mg/dL)	2.1/ 2.1	ND/ND	1.8/ND	1.5/ 1.8	2.3/ND	1.8/ 2.2	2.0/ 2.4	2.2/ 1.8	2.1/ 2.4	2.0/ND	1.9/ 2.0

Table 2

	Cisplatin dose	Δ Plasma creatinine	Δ Plasma urea	Δ Calcemia	Δ Magnesemia	Δ NGAL	Δ GM2AP
Cisplatin dose		0.127 (0.786)	-0.110 (0.814)	0.115 (0.807)	-0.338 (0.458)	0.207 (0.556)	0.456 (0.159)
Δ Plasma creatinine			0.893 (0.007)**	0.000 (1.000)	-0.821 (0.023)*	0.250 (0.589)	0.643 (0.119)
Δ Plasma urea				-0.406 (0.365)	-0.779 (0.039)*	0.131 (0.780)	0.474 (0.282)
Δ Calcemia					0.633 (0.127)	-0.464 (0.295)	0.100 (0.831)
Δ Magnesemia						-0.611 (0.145)	-0.525 (0.227)
Δ NGAL							0.146 (0.687)
Δ GM2AP							

Figure 1

Click here to access/download;Figure;figure 1.jpg

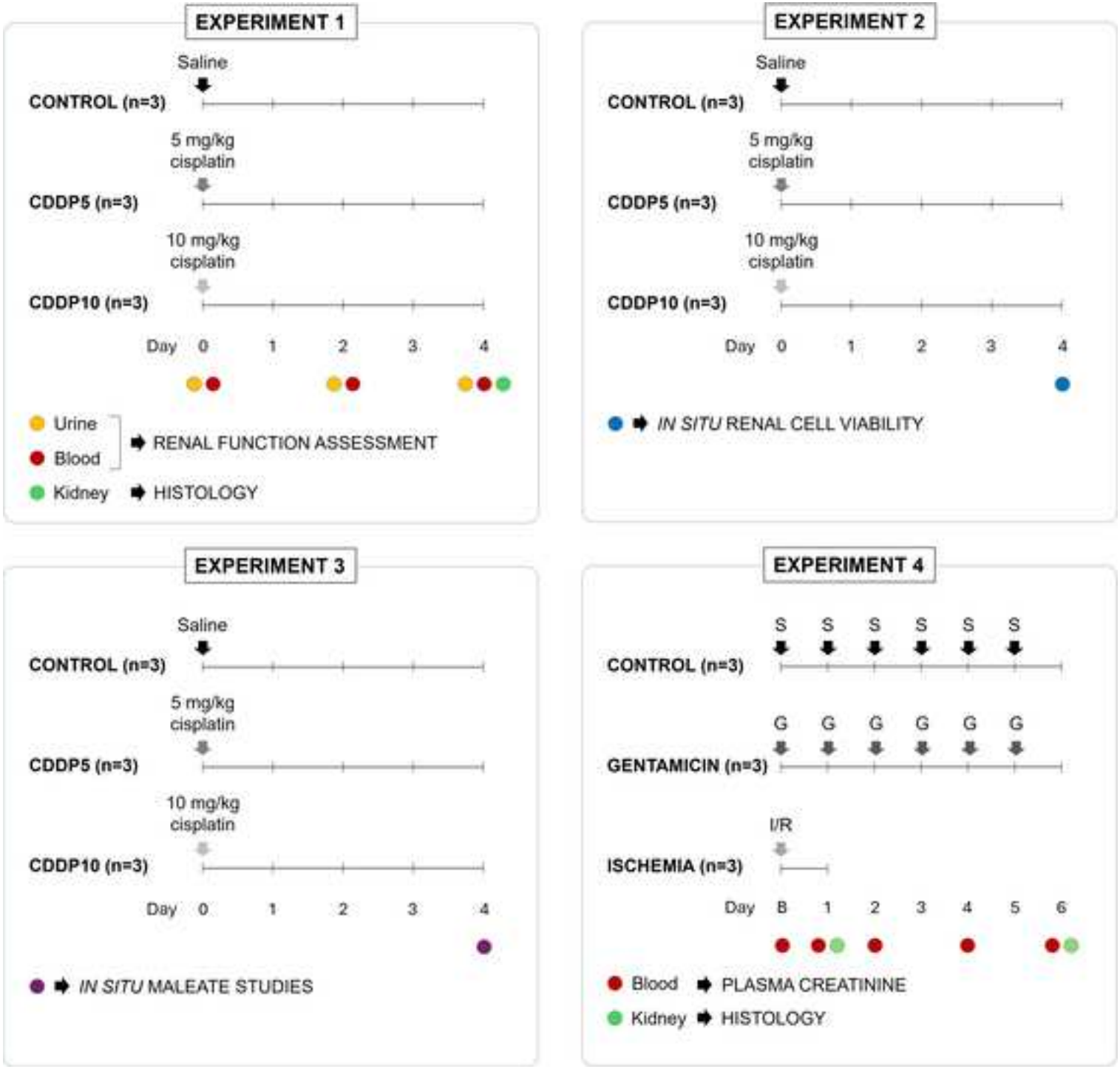


Figure 2

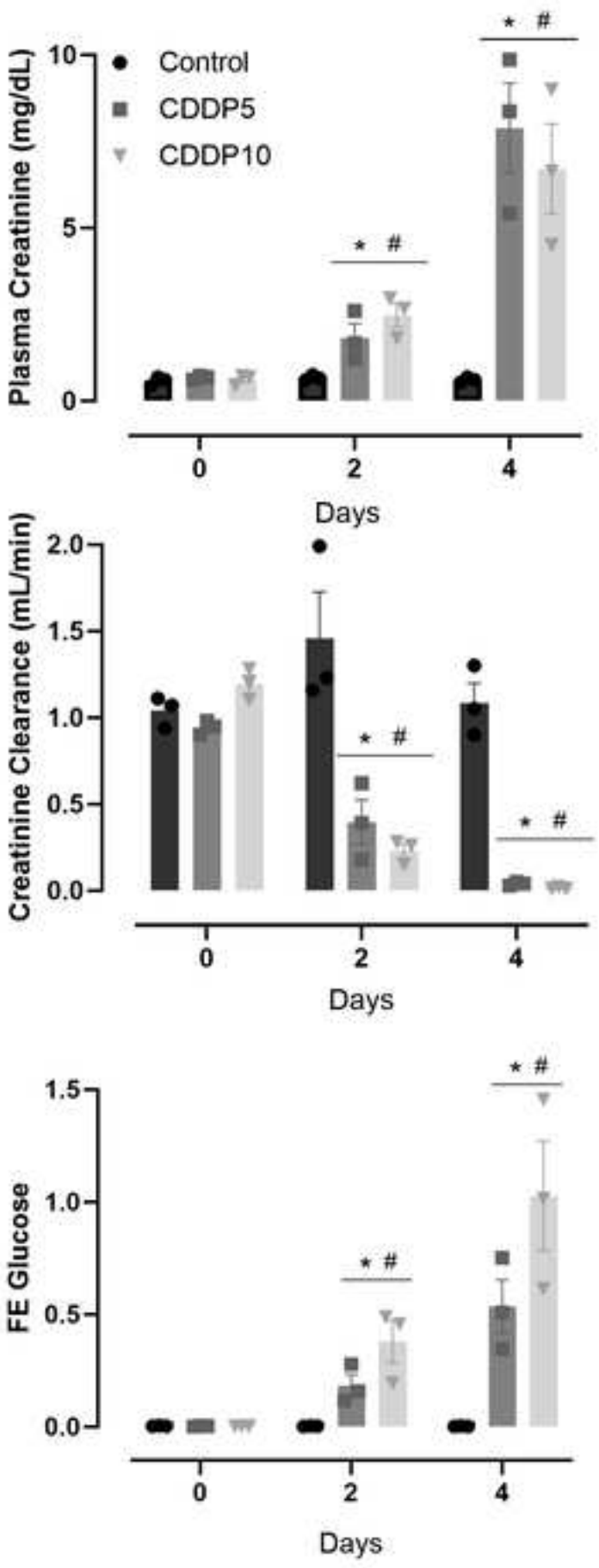


Figure 3

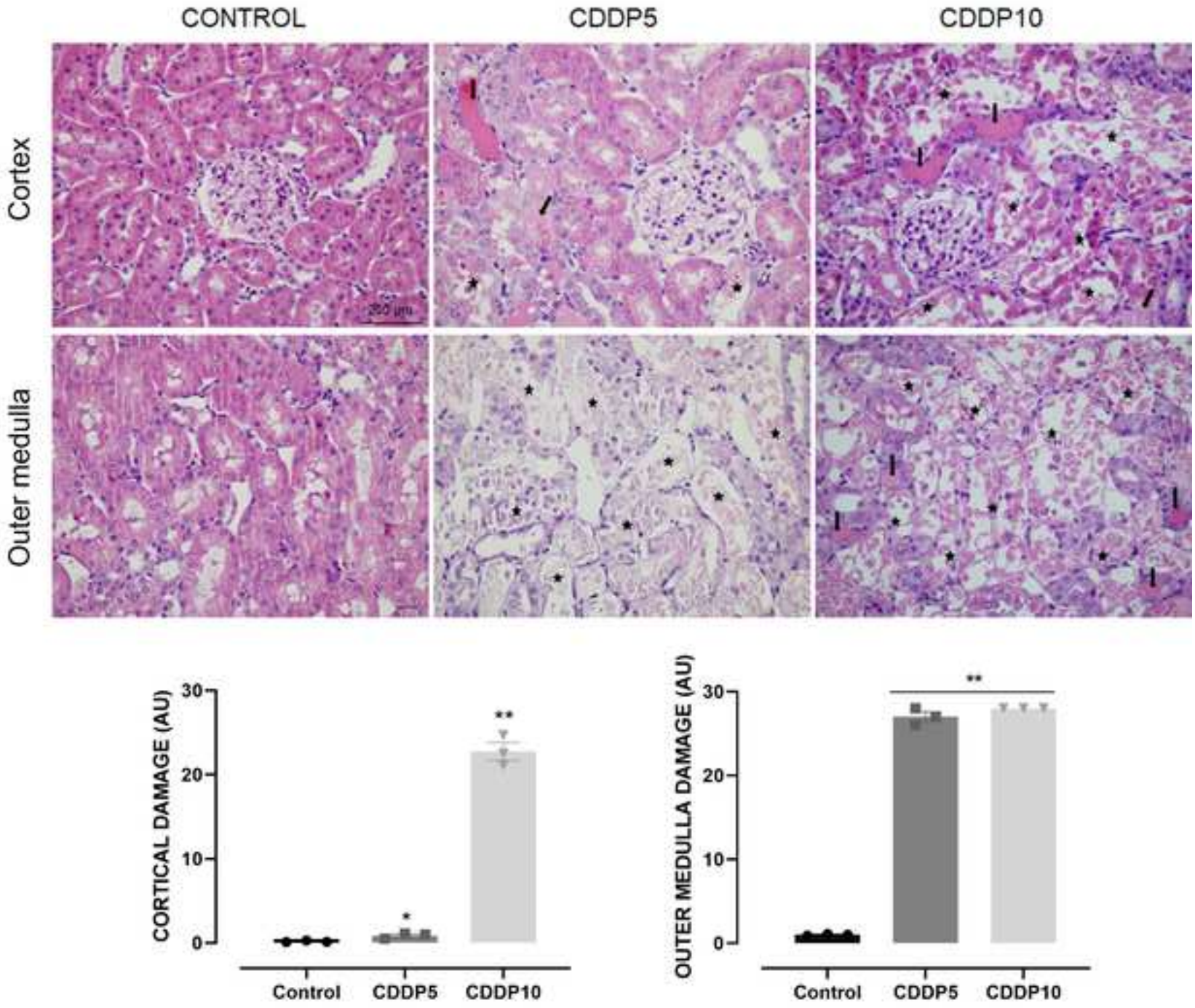


Figure 4

Click here to access/download;Figure;figure 4.jpg

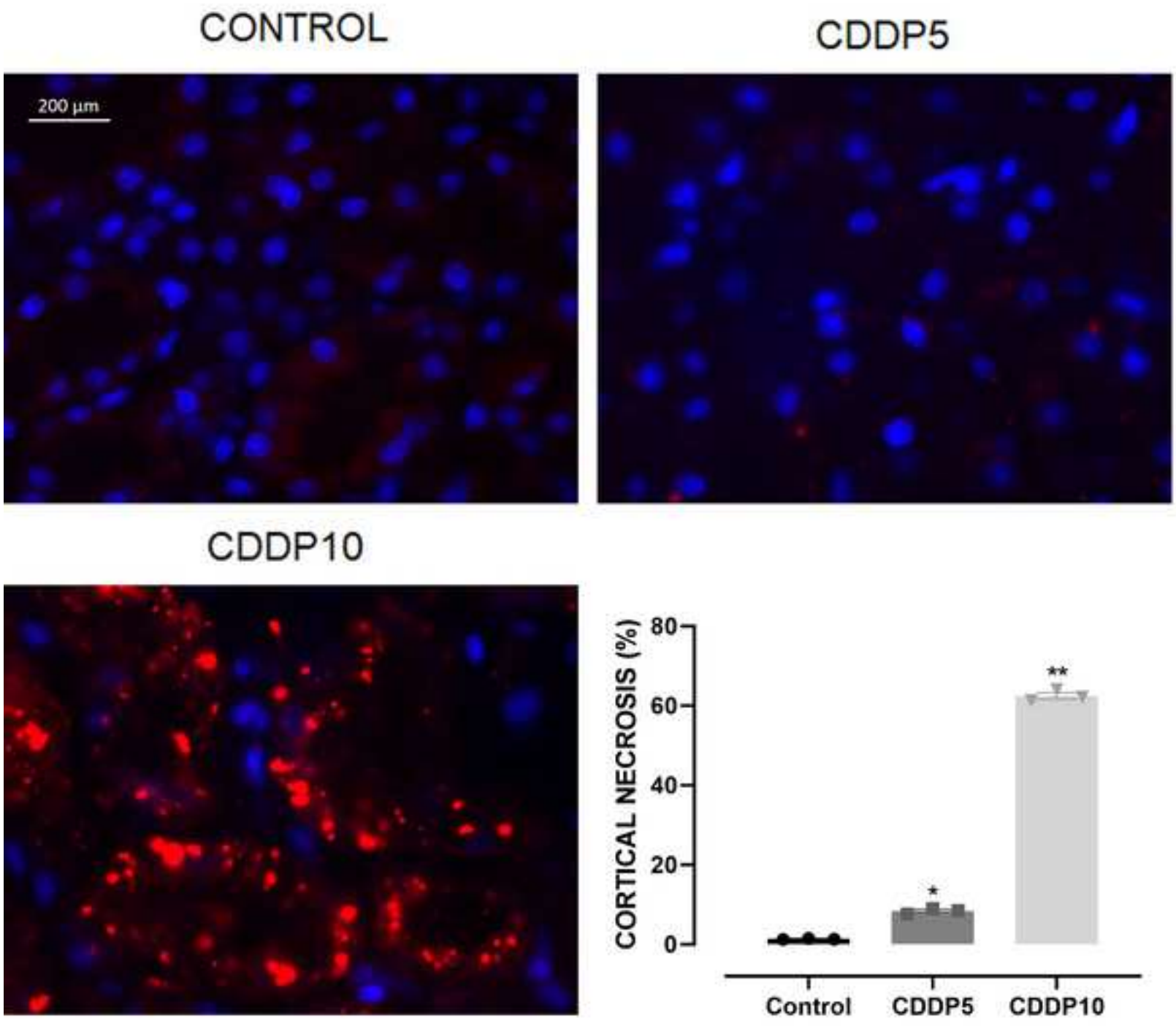


Figure 5

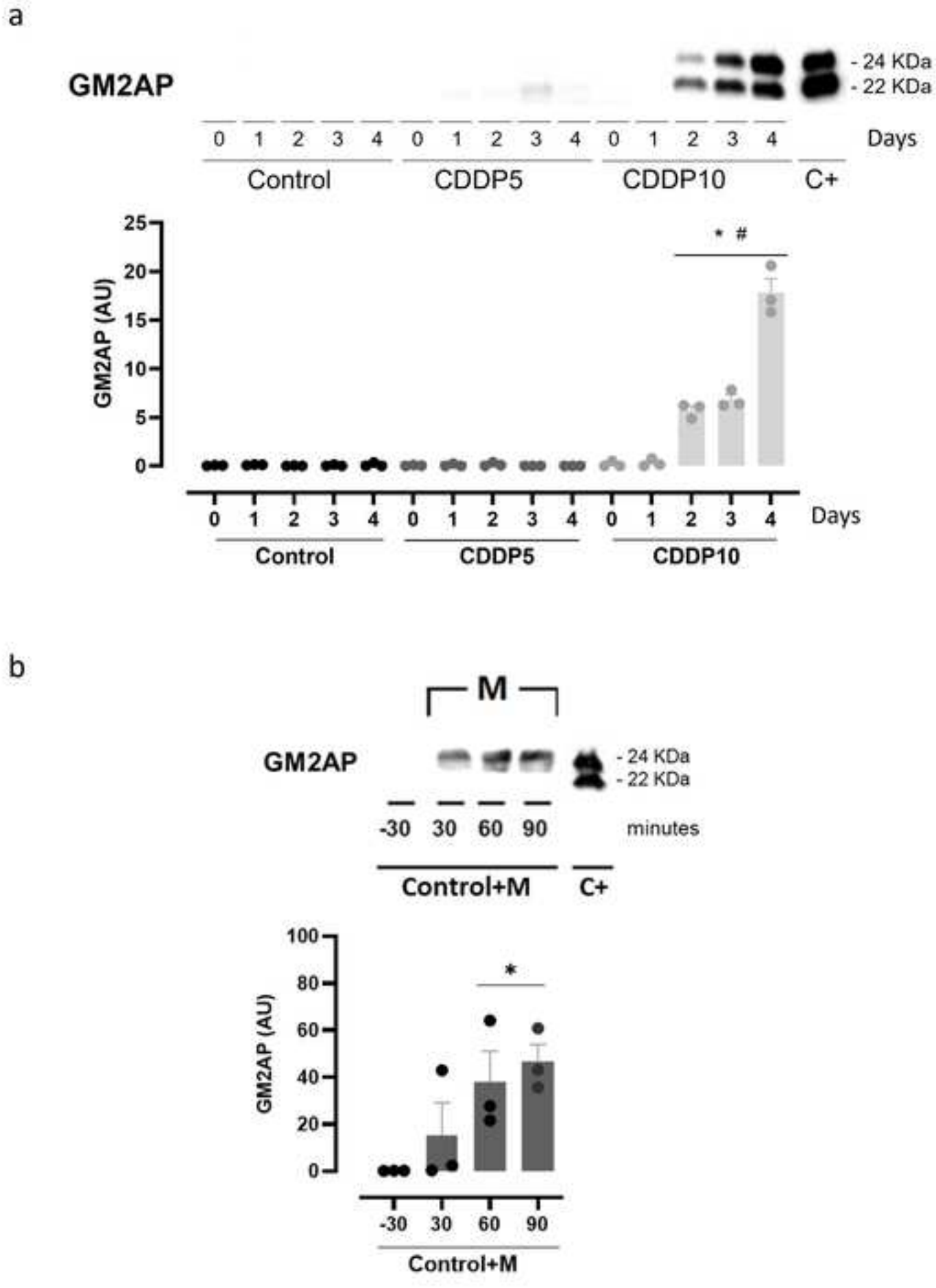


Figure 6

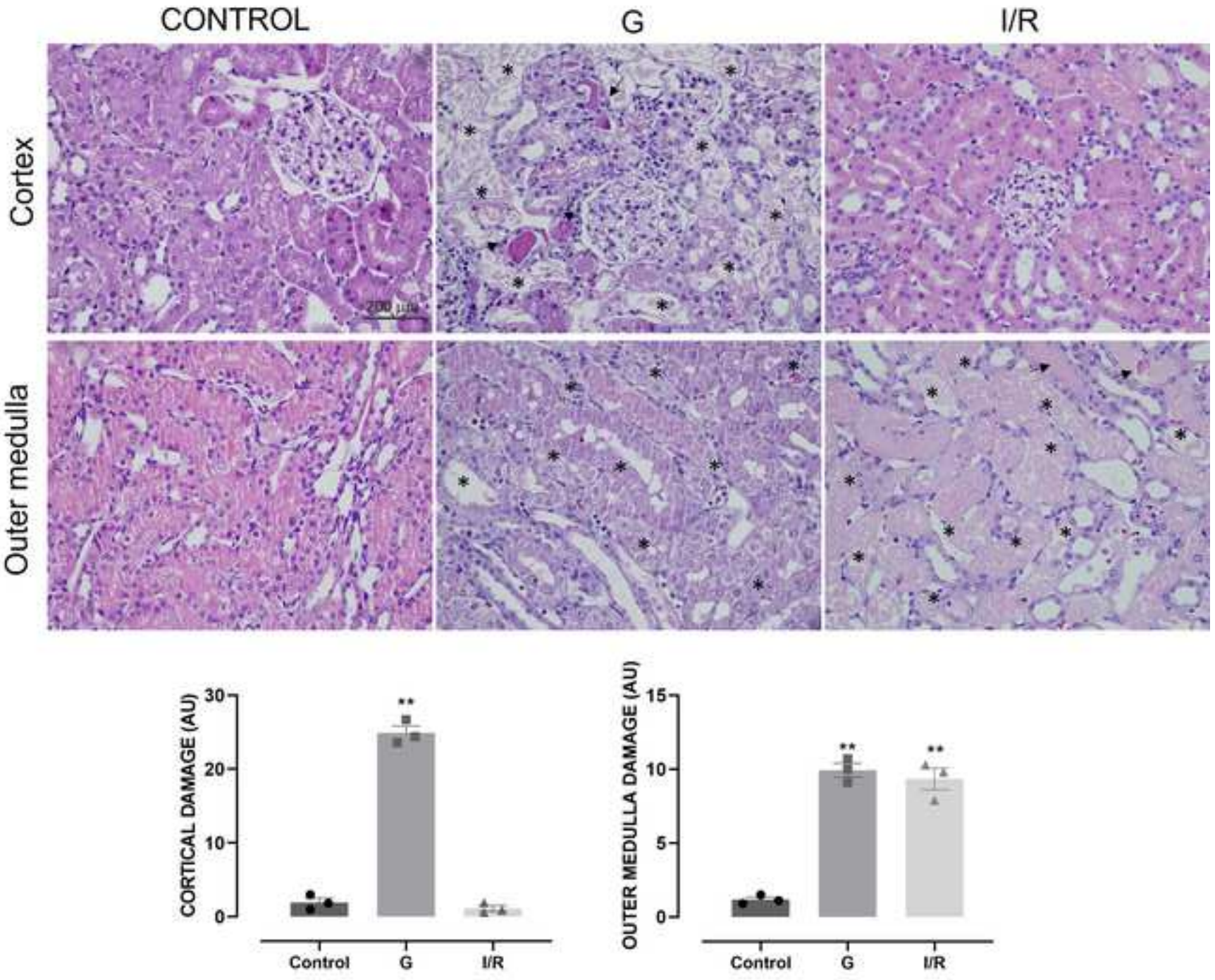


Figure 7

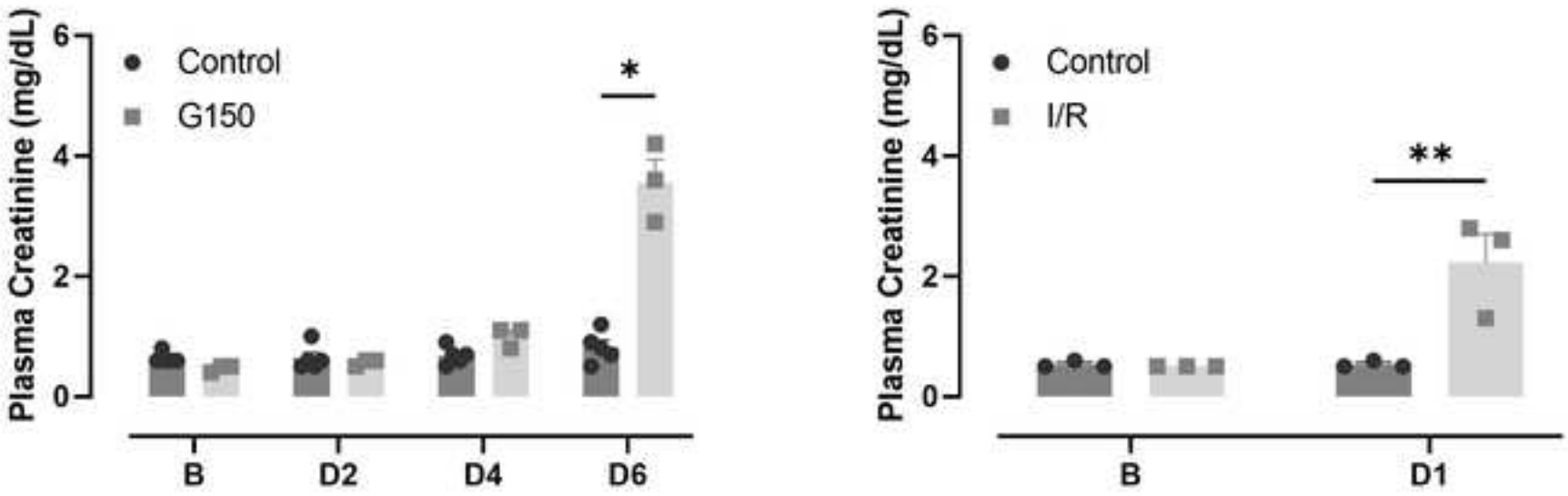
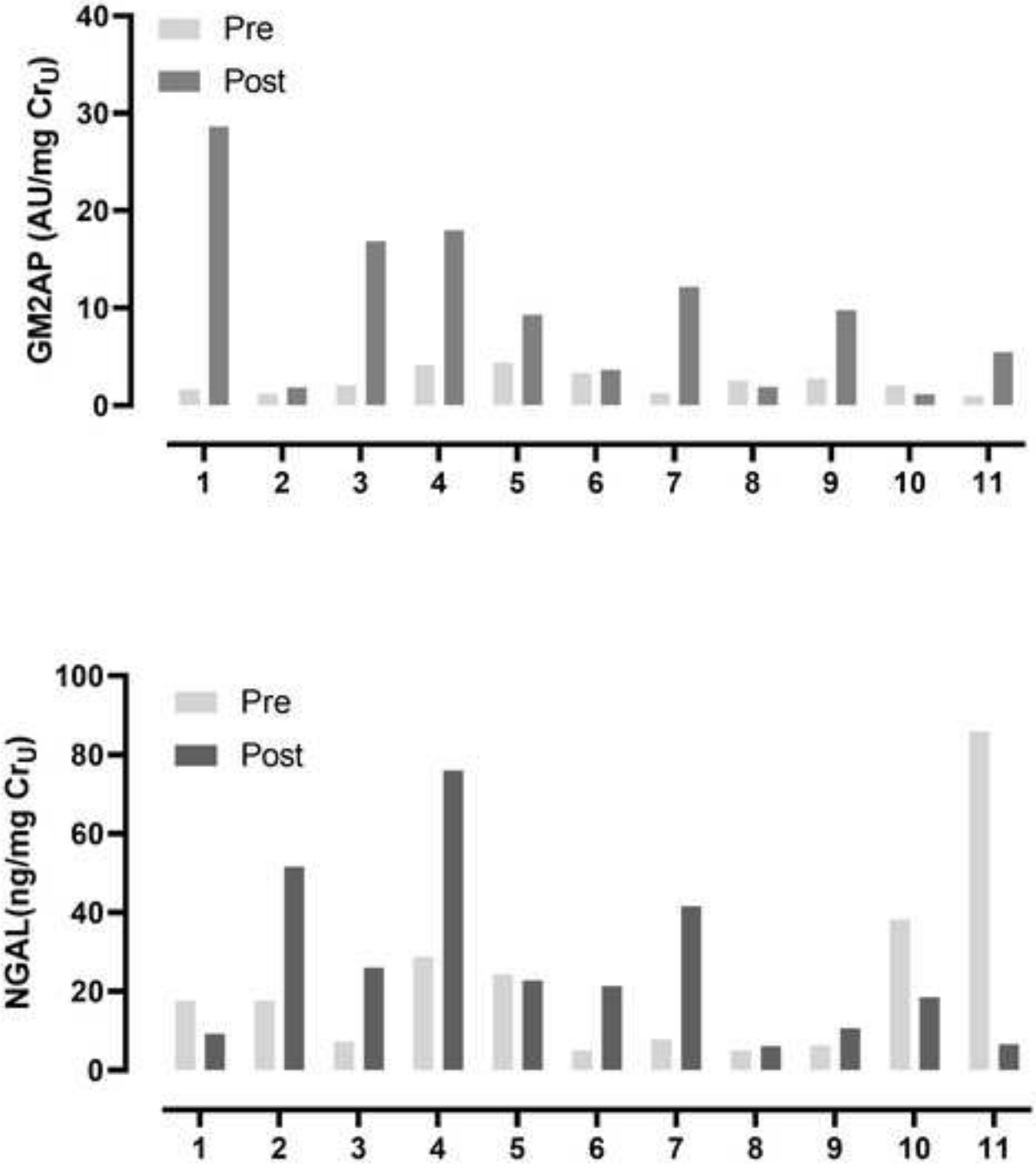


Figure 8



Declaration of interests

☒ 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.

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