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Urinary transferrin pre-emptively identifies the risk of renal damage posed by subclinical tubular alterations

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ABSTRACT

Nephrotoxicity is an important limitation to the clinical use of many drugs and contrast media. Drug nephrotoxicity occurs in acute, subacute and chronic manifestations ranging from glomerular, tubular, vascular and immunological phenotypes to acute kidney injury. Pre-emptive risk assessment of drug nephrotoxicity poses an urgent need of precision medicine to optimize pharmacological therapies and interventional procedures involving nephrotoxic products in a preventive and personalized manner. Biomarkers of risk have been identified in animal models, and risk scores have been proposed, whose clinical use is abated by their reduced applicability to specific etiological models or clinical circumstances. However, our present data suggest that the urinary level of transferrin may be indicative of risk of renal damage, where risk is induced by subclinical tubular alterations regardless of etiology. In fact, urinary transferrin pre-emptively correlates with the subsequent renal damage in animal models in which risk has been induced by drugs and toxins affecting the renal tubules (i.e. cisplatin, gentamicin and uranyl nitrate); whereas transferrin shows no relation with the risk posed by a drug affecting renal hemodynamics (i.e. cyclosporine A). Our experiments also show that transferrin increases in the urine in the risk state (i.e. prior to the damage) precisely as a consequence of reduced tubular reabsorption. Finally, urinary transferrin pre-emptively identifies subpopulations of oncological and cardiac patients at risk of nephrotoxicity. In perspective, urinary transferrin might be further explored as a wider biomarker of an important mechanism of predisposition to renal damage induced by insults causing subclinical tubular alterations.

1. Introduction

Drug nephrotoxicity is a most serious health problem [1,2] and an important cause of failure along the drug discovery process [3]. Members of many drugs families including antibiotics, antineoplastics, antivirals, immunosupressors, proton pump inhibitors, analgesics, antihypertensives and others; radiological iodinated contrast media; and 22 % of the 100 most used drugs in intensive care are nephrotoxic [4]. Drug nephrotoxicity shows a wide range of acute, subacute and chronic manifestations depending on the mechanisms, dosage, and patient

Abbreviations: AKI, acute kidney injury; ATN, acute tubular necrosis; CIN, contrast-induced nephropathy; CL_{cr}, creatinine clearance; Cr_{pl}, plasma creatinine; Cr_u, urinary creatinine; FAA, fumarylacetoacetase; GM2AP, ganglioside M2 activator protein; IGFBP-7, insulin-like growth factor binding protein-7; KIM-1, kidney injury molecule 1; NAG, N-acetylglucosaminidase; NGAL, gelatinase-associated lipocalin; ROC, Receiver Operating Characteristic; SEM, standard error of the mean; TIMP-2, tissue inhibitor of metalloproteinase-2; UF, urine flow

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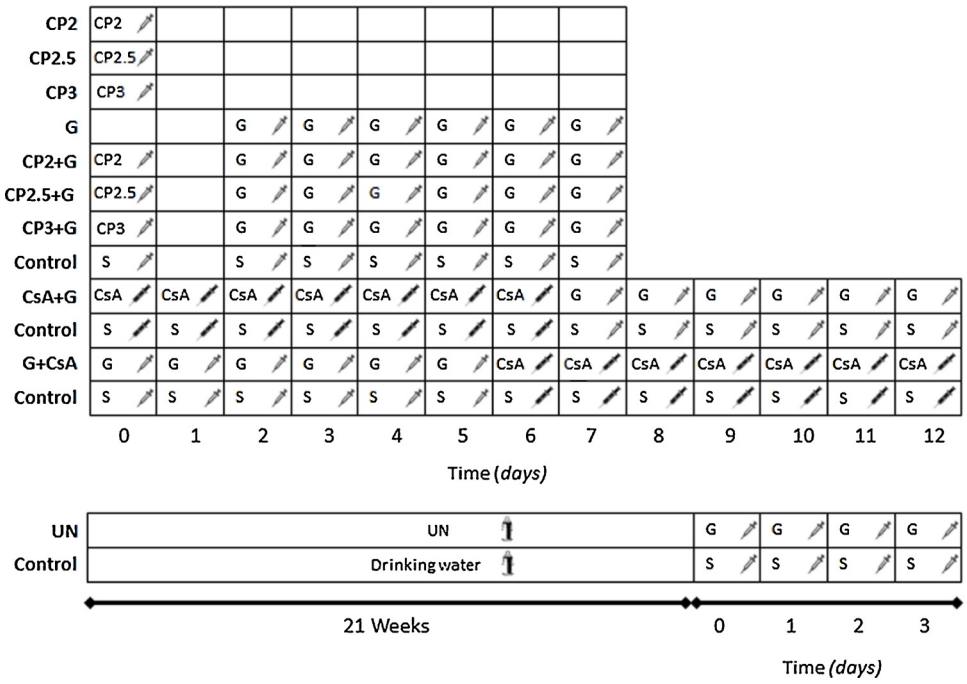

Fig. 1. Schematic representation of the experimental groups. White syringes symbolize intraperitoneal administration, and black syringes subcutaneous administration. Bottles represent oral administration in drinking water. CP, cisplatin; 2, 2.5 and 3 refers to the dose (mg/kg); G, gentamicin (50 mg/kg); S, saline; CsA, cyclosporine (15 mg/kg); UN, uranyl nitrate (5.4 g/L).

characteristics and comorbidities, reflecting damage or functional alterations to different nephron segments and systemic and renal hemodynamics. The clinical manifestations of nephrotoxicity often go unrecognized, especially during short exposures, which hinders diagnosis, severity assessment and evaluation of immediate, medium- and long-term consequences. Recently, an international initiative has generated a standard consensus definition of drug nephrotoxicity [5]. Specifically, several phenotypes of drug nephrotoxicity were recognized, namely acute kidney injury (AKI) due to acute tubular necrosis (ATN), tubular disorders, glomerular disorders, nephrolithiasis and combinations thereof. In fact, these latter may be independent nephrotoxic manifestations, pose a subclinical degree of AKI, or even be the cause and underlie an AKI. Additional phenotypes, not included in this definition, are hemodynamic AKI [2], nephritis [6] and vascular and microvascular angiopathies [7].

AKI is a syndrome of sudden renal excretory dysfunction with serious health consequences and high cost [8], consuming 1 % of total health budget [9] and 5 % of hospital expenditure [10,11]. According to international scales (i.e. RIFLE, AKIN and KDIGO), AKI is diagnosed by increments in plasma creatinine (Cr_{pl}) [12]. However, Cr_{pl} may not be sufficiently sensitive to detect acute nephrotoxicity [13], because significant damage must occur for Cr_{pl} to increase. A Cr_{pl} insensitivity window results from the recruitment of renal functional reserve and from an increased creatinine secretion [14]. More sensitive and earlier biomarkers are under development for the detection of subclinical AKI, i.e. with normal Cr_{pl} [15,16], including neutrophil gelatinase-associated lipocalin (NGAL), kidney injury molecule 1 (KIM-1), tissue inhibitor of metalloproteinase-2 (TIMP-2) and insulin-like growth factor binding protein-7 (IGFBP-7) [17], and N-acetylglucosaminidase (NAG) [18]. Tubular disorders encompass alterations in tubular transport leading to mishandling of phosphate, glucose, magnesium, potassium, proteins or water [5].

Following this international definition, a 6R framework (risk, recognition, response, renal support, rehabilitation and research) has been proposed to identify and manage drug induced renal damage, where early and sensitive diagnosis is critical for effective patient triage and clinical handling, and for optimal outcome [1]. In the line of risk assessment, acquired predisposition to renal damage has been described as a distinct but complementary concept to early and subclinical damage. Predisposition is a condition of increased risk of suffering renal damage, hitherto undetectable at the clinical level. Only population risk factors have been identified, which include age, previous renal function, diabetes, volemia, and others [2]. However, stratification of patients according to their individual risk, before being exposed to kidney-injuring medical or environmental conditions may open a new concept to handle AKI from a truly preventive and personalized manner, and also for monitoring AKI risk in the community. This concept has been mainly developed hitherto in animal models of nephrotoxicity. [19–21], in which animals are treated with subtoxic regimes of different nephrotoxic drugs. These treatments render animals, compared to untreated controls, more susceptible to developing an AKI; so that when they are subject to a second aggression (completely innocuous for controls), an overt AKI (i.e. acute tubular necrosis) occurs.

Biomarkers of predisposition have been identified in these animal models, which include t-gelsolin, ganglioside M2 activator protein (GM2AP), fumarylacetoacetase (FAA), and others. However, clinical application has been limited by their specificity for individual predisposing agents. For instance, no common mechanism of predisposition has been identified, and no universal marker of increased risk is hitherto known. In fact, patient-stratifying scores have been developed, but only with *ad hoc* and limited applicability to specific clinical scenarios [22]. In this article we show evidence suggesting that urinary transferrin might be a wider biomarker of predisposition to renal damage induced by insults causing subclinical tubular alterations, which pre-emptively identifies a subpopulation of oncological and cardiac patients at risk of nephrotoxicity.

2. Material and methods

Except where otherwise indicated, all reagents were purchased from Sigma–Aldrich (Madrid, Spain).

2.1. Animal models and experimental protocols

Male Wistar rats (200 − 250 g) were maintained under controlled conditions within the University of Salamanca Animal House facility, with free access to water and standard chow. All procedures were approved by the Bioethics Committee of the University of Salamanca and the Consejería de Agricultura y Ganadería of the Junta de Castilla y León. 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. At specific time points, rats were placed in individual metabolic cages for urine collection.

Rats were divided into the following groups (n = 4 per group) (Fig. 1): CP2, CP2.5 and CP3 groups, that received an intraperitoneal (i.p.) injection of a sub-nephrotoxic dose of cisplatin (2, 2.5 and 3 mg/kg, respectively); CP2 + G, CP2.5 + G and CP3 + G groups, which received a sub-nephrotoxic dose of cisplatin (as above), followed by a sub-nephrotoxic dose of gentamicin (50 mg/kg i.p. for 6 days from day 2), as the second nephrotoxic agent; G group, that received gentamicin (50 mg/kg i.p.) for 6 days; CsA + G, which received a sub-nephrotoxic dose (15 mg/kg) of subcutaneous (s.c.) cyclosporine for 7 days, and gentamicin (50 mg/kg i.p.) for 6 days from day 7; G + CsA, which received gentamicin (50 mg/kg i.p.) for 6 days, and cyclosporine (15 mg/kg s.c.) for 7 days from day 6; UN + G, which received uranyl nitrate (5.4 g/L in the drinking water) for 21 weeks, and gentamicin (50 mg/kg i.p.) for 4 days from week 21; and their respective Control groups, which received the vehicle (i.e. saline). 24 h urine was collected in metabolic cages, cleared by centrifugation and stored at -80 °C. Blood samples (200 μL) were obtained in heparinized capillaries from a small incision in the tail tip. Plasma was separated by centrifugation and kept at -80 °C. At the end, rats were anesthetized, blood was collected and the kidneys dissected and fixed in 3.7 % para-formaldehyde for histological studies.

In order to establish whether transferrin is reabsorbed in the proximal tubule under physiological conditions, sodium maleate was administered at a dose of 400 mg/kg intravenously to 6 healthy rats to cause decoupling of their tubular transport systems. Urine samples were directly collected from the bladder, before and after administration of maleate.

2.2. In situ renal perfusion

At the end of the experimental protocol of treatment with cisplatin, some rats were anesthetized and an extracorporeal circuit for kidney perfusion was set up, as described elsewhere [23]. Briefly, the renal artery and vein of the left kidney and the urinary bladder were cannulated. Renal perfusion was interrupted during surgery for a maximum of one minute. Oxygenated and warm (37 °C) Krebs-dextran [40 g/L of dextran (molecular weight 64 − 76 kDa) in Krebs solution (118.3 mM NaCl, 4.7 mM KCl, 1.8 mM $CaCl_2$, 1.2 mM $MgSO_4$, 1.2 mM KH_2PO_4, 25 mM $NaHCO_3$, 0.026 mM EDTA, 11.1 glucose, pH = 7.4)] was perfused through the renal artery at 3 mL/min. Urine fractions were collected from a catheter placed in the urinary bladder, starting before initiating the perfusion with Krebs (when the kidney was still perfused with blood), and for 60 min after initiating Krebs perfusion. As a control of these perfusion experiments, in order to exclude a potential artefact derived from surgery or other experimental maneuvers, kidneys were also perfused *in situ* with blood from the carotid artery. For this purpose, the artery, vein and ureter of the left kidney were ligated as above. A catheter was placed in the left carotid artery and connected directly to the renal artery. Urine was collected as above.

2.3. Renal function assessment in animal models

Plasma and urine creatinine were measured using a commercial kit based on the Jaffe method [24] (*QuantiChrom Creatinine Assay Kit*, BioAssay Systems, Hayward, CA, USA). Plasma urea was determined using a commercial kit based on the Jung method [25] (*QuantiChrom Urea Assay Kit*, BioAssay Systems, Hayward, CA, USA). Creatinine clearance (CL_{cr}) was calculated using the formula: $CL_{cr} = Cr_{ur}$ x UF/Cr_{pl}; where Cr_u corresponds to urinary concentration of creatinine, UF is urine flow and Cr_{pl} is plasma concentration of creatinine. Proteinuria was measured with the Bradford assay [26].

2.4. Renal histology in animal models

Tissue was fixed in 3.7 % para-formaldehyde and embedded in paraffin; 5 μm tissue sections were stained with hematoxylin and eosin. Photographs were taken under an Olympus BX51 microscope connected to an Olympus DP70 color, digital camera (Olympus, Madrid, Spain).

2.5. Patients and clinical protocol

Two observational studies were conducted to pre-emptively evaluate the risk of renal damage in two, non-related clinical scenarios. Both were proof-of-concept studies rooted in routine clinical practice, and not ad-hoc designed clinical trials, and followed the principles established in 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. Both studies were approved by the Ethical Committee for Clinical Investigation and all patients provided written informed consent. In both studies exclusion criteria were any diseases or clinical conditions that, in the opinion of the investigators, would interfere with the study evaluation.

2.5.1. Study 1: oncology patients treated with platinated antineoplastics

This study was conducted during 2015 and 2016 with patients from the Medical Oncology Service of the University Hospital of Salamanca (Salamanca, Spain). Patients diagnosed with various types of cancer and prescribed cisplatin or carboplatin, were included. Blood and urine samples were collected immediately before and 72 h after the administration of each chemotherapy cycle including cisplatin or carboplatin.

Platinated antineoplastics are effective chemotherapeutic drugs [27–29] commonly used to treat a variety of solid cancers, including head and neck, oesophageal, non-small cell lung, testicular, ovarian, breast, cervical and bladder cancer [30]. Their dosage and effectiveness are limited by their nephrotoxicity, which occurs in 25–35 % of adult [31] and in 70 % of paediatric [32] patients, and exhibits a tubular phenotype, ranging from electrolytic disturbances derived from impaired tubular handling (i.e. most typically hypomagnesemia and hypokalemia), to ATN and AKI (with rising plasma creatinine and urea levels) [33–36]. Accordingly, evidence of nephrotoxicity was defined as detection of abnormalities after the administration of the platinum-based antineoplastic in at least two of the following plasma analytes simultaneously, defined as: a) an increase in plasma creatinine over the normal range; b) an increase in plasma urea of over the normal range; c) a decrease in calcemia below the normal range; and d) a decrease in magnesemia below the normal range. Normal ranges for these parameters are shown in Table 1 caption. Patients were divided into two groups: (i) Cases, i.e. patients who underwent alteration of at least two of these four blood parameters evaluated at some point during their treatment; and (ii) Controls, i.e. patients who did not suffer alterations in those parameters after at least three cycles of chemotherapy. Plasma creatinine, urea, calcium and magnesium were obtained from routine blood tests. Basal urine and urine previous to the moment of peak alteration of these blood parameters were selected for biomarker assessment.

2.5.2. Study 2: cardiology patients exposed to iodinated contrast media

Iodinated contrast media cause a specific type of acute kidney injury known as contrast-induced nephropathy (CIN), defined as an increase in the plasma creatinine concentration greater than 0.5 mg/dl, or 25–50 % of the baseline value, within the first 5 days after the administration of the contrast medium (in the absence of other causes that justify it, such as surgery, other nephrotoxic drugs, hypovolemia, etc.) [37,38]. The incidence of CIN in patients undergoing percutaneous coronary

Table 1
Initial characteristics and pharmacological data of patients included in the study. Values are expressed as mean ± SEM. n.s.: non-significant. Normal value ranges (mg/dL) for biochemical parameters: 1) Plasma creatinine (0.7–1.2 in males / 0.5-0.9 in females). 2) Plasma urea (16.6–48.5). 3) Calcemia (8.8–10.2 / 8.6–10.0 in females). 4) Magnesemia (1.6–2.4 in males / 1.6–2.6 in females).

STUDY 1: ONCOLOGY

Anthropometric and pharmacological data

	Cases (n = 18)		Controls (n = 17)		p-value
Gender (m/f)	13/5		9/8		n.s.
Age (years)	61.1 ± 1.9		57.1 ± 1.6		n.s.
Weight (kg)	78.1 ± 3.3		71.2 ± 2.7		n.s.
Body Mass Index (kg/m^2)	28.6 ± 1.1		26.4 ± 1.3		n.s.
Body Surface (m^2)	1.82 ± 0.04		1.78 ± 0.03		n.s.
Patients treated with cisplatin/carboplatin	14/4		9/8		n.s.
Cisplatin dose (mg)	107 ± 11		99 ± 13		n.s.
Carboplatin dose (mg)	535 ± 56		625 ± 41		n.s.
Biochemical data					
	Men	Women	Men	Women	
Plasma creatinine (mg/dL)	0.86 ± 0.06	0.67 ± 0.12	0.72 ± 0.07	0.58 ± 0.06	n.s.
Plasma urea (mg/dL)	39.38 ± 3.82	44.80 ± 5.43	30.22 ± 2.77	28.25 ± 6.46	n.s.
Calcemia (mg/dL)	9.09 ± 0.23	8.64 ± 0.42	9.19 ± 0.18	9.40 ± 0.29	n.s.
Magnesemia (mg/dL)	1.97 ± 0.12	2.18 ± 0.08	1.97 ± 0.08	2.13 ± 0.11	n.s.

STUDY 2: CARDIOLOGY

Anthropometric and pharmacological data

	Cases (n = 31)		Controls (n = 122)		p-value
Gender (m/f)	22/9		93/29		n.s.
Age (years)	78.0 ± 2.3		72.6 ± 1.2		p = 0.009
Weight (kg)	69.9 ± 2.1		75.6 ± 1.2		p = 0.042
Body Mass Index (kg/m^2)	26.4 ± 0.8		27.8 ± 0.4		p = 0.044
Patients treated with iohexol/iodixanol/no data	5/22/4		27/84/11		n.s.
Iohexol dose (mg)	334.6 ± 46.7		263.9 ± 24.1		n.s.
Iodixanol dose (mg)	291.4 ± 23.5		288.7 ± 16.3		n.s.
Biochemical data					
	Men	Women	Men	Women	
Plasma creatinine (mg/dL)	1.39 ± 0.15	1.14 ± 0.26	1.11 ± 0.53	0.89 ± 0.05	n.s.
Plasma urea (mg/dL)	72.75 ± 9.11	56.37 ± 7.19	49.81 ± 2.52	50.42 ± 4.20	Men p = 0.005 Women n.s.

interventions can reach 25 % [39,40] or 50 % in high-risk patients [41,42]. CIN is the third most frequent cause of acute renal failure in hospitalized patients [43].

This study was conducted from 2015 to 2017 with patients from the Cardiology Department of the University Hospital of Salamanca (Salamanca, Spain). Patients undergoing cardiac catheterization involving administration of iodinated contrast media (iohexol or iodixanol) were included. Blood and urine samples were collected immediately before the administration of the contrast media, and daily after until discharge. Patients were classified as Cases or Controls if subsequently underwent CIN or not, respectively. Accordingly, (i) Cases were patients who suffered CIN; and (ii) Controls were patients who did not suffer CIN. Plasma creatinine and urea were obtained from routine blood tests, both at baseline and during follow-up. Basal urine was selected for biomarker assessment.

2.6. Quantification of biomarkers of early kidney damage in rat and human urine samples

NAG activity was quantified using a commercial kit [*N-Acetyl-β-D-glucosaminidase (NAG) assay kit*, Diazyme, Poway, CA, USA] following the manufacturer's instructions. NGAL was measured by commercial ELISAs (*Human NGAL ELISA Kit 036CE* and *Rat NGAL ELISA Kit 046*, BioPorto Diagnostics, Hellerup, Denmark), according to the manufacturer's instructions. Biomarker values in humans were factored by urinary creatinine concentration, as a means to normalize the effect of urine concentration.

2.7. Quantification of transferrin in rat and human urine samples

Transferrin was measured by Western blot. Briefly, 21 μL per human urine sample, or a volume of urine from each rat corresponding to the same fraction of 24 -h urinary output, were separated by 4–20 % gradient polyacrylamide gel electrophoresis (*4-20 % Criterion TGX Stain-Free Protein Gel*, Bio-Rad Laboratories, Hercules, CA, USA). Immediately, proteins were electrically transferred to an Immun-Blot PVDF Membrane (Bio-Rad Laboratories, Hercules, CA, USA) and incubated with anti-transferrin antibody (sc-22597, Santa Cruz Biotechnology, Santa Cruz, CA, USA), followed by horseradish peroxidase-conjugated secondary antibodies and chemiluminescent detection (Clarity Western ECL Substrate, Bio-Rad Laboratories, Hercules, CA, USA) with photographic films (Fujifilm, Tokyo, Japan). Bands were

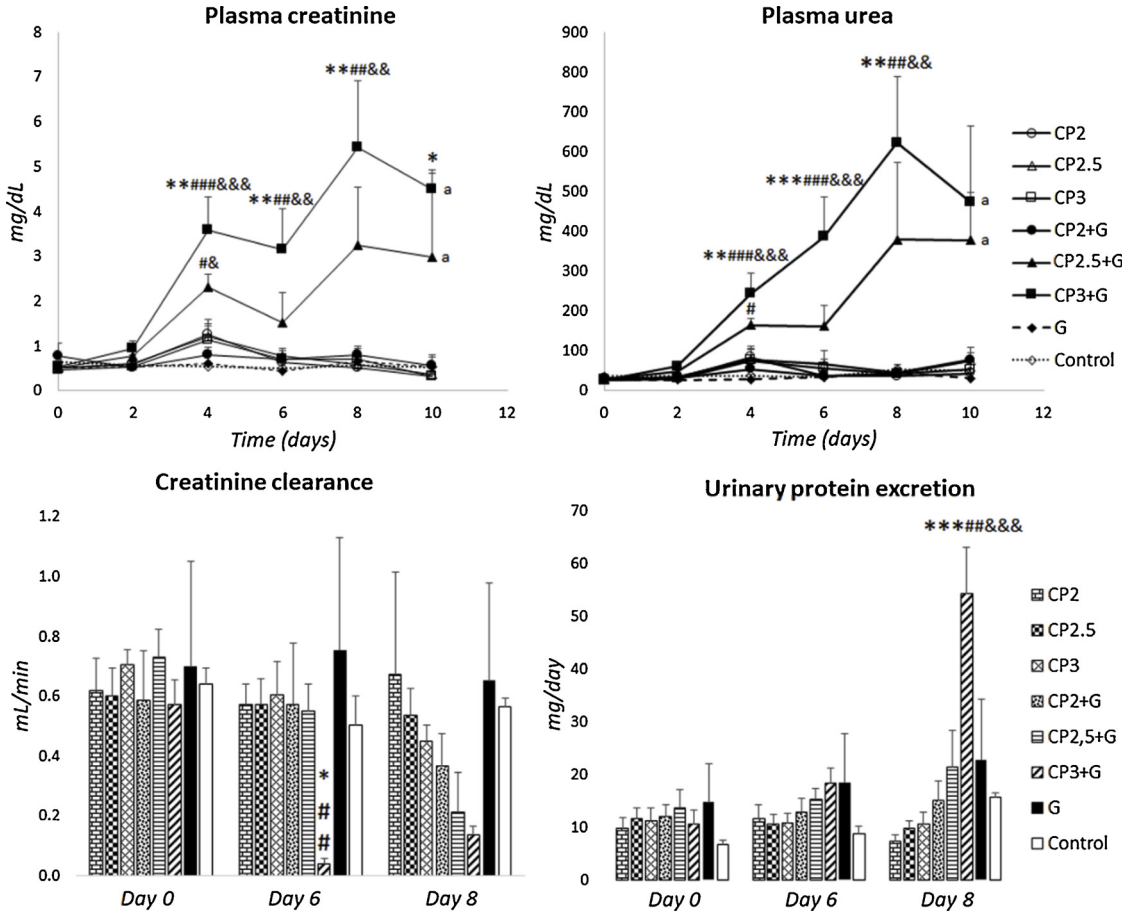

Fig. 2. Plasma creatinine and urea levels, creatinine clearance and urinary protein excretion in a rat model of predisposition to AKI by cisplatin. Values are expressed as the mean ± SEM. a: There was a death before completing the experiment; *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$ vs. its corresponding group without gentamicin; #, $p < 0.05$; ##, $p < 0.01$; ###, $p < 0.001$ vs. G group; &, $p < 0.05$; &&, $p < 0.01$; &&&, $p < 0.001$ vs. Control group. CP, cisplatin; G, gentamicin.

quantified by densitometry analysis with the Scion Image software (Frederick, MD, USA). Inter-gel normalization was carried out by referring band quantification data to the same positive control loaded in each gel. Transferrin values in humans (arbitrary units, AUs) were factored by urinary creatinine concentration, as a means to normalize the effect of urine concentration [44].

2.8. Statistical analysis

Data are presented as the mean ± standard error of the mean (SEM). Data normal distribution was evaluated using the Kolmogorov-Smirnov test. After that, an ANOVA with Tukey's test was performed to compare preclinical experimental groups; and paired Chi-Square (for qualitative variables), Student's *t*-test or U-Mann-Whitney (for quantitative variables) tests were also used to compare some preclinical results and to assess differences between Cases and Controls in the clinical study. In preclinical studies, the correlation between the urinary levels of each biomarker before the administration of the second nephrotoxic agent versus the severity (plasma urea and creatinine) of subsequent AKI was evaluated by the Pearson's test. In the clinical study, the diagnostic capacity of transferrin was evaluated through a Receiver Operating Characteristic (ROC) curve-based analysis [45]. Statistical analysis was performed with the IBM SPSS Statistics 20.0 software (International Business Machines, Armonk, NY, USA). Microsoft Office Excel 2016 and IBM SPSS Statistics 20.0 were used to create the artwork and illustrations presented.

3. Results

3.1. Urinary transferrin is a biomarker of predisposition to AKI in specific animal models of subclinical tubular damage and not in others

Transferrin had been previously identified as a urinary biomarker of uranium-induced predisposition to AKI [20], but it had not been tested in other preclinical models, and it was thus unknown whether it was stimulus-specific. The purpose of this new study was to elucidate whether urinary transferrin marks a specific process or mechanism of predisposition to AKI, is thus a common marker of different predisposing causes, or at least is a marker of a common mechanism engaged by different predisposing causes. Figs. 2, 3 and 4 show that renal function and renal histology become altered only in animals previously predisposed to AKI by subnephrotoxic regimes of cisplatin, cyclosporine, gentamicin and uranyl nitrate (i.e. the different predisposing agents), and not in non-predisposed animals, when subsequently challenged by a subnephrotoxic dose of a second toxin (i.e. the triggering insult). In fact, exposure to only the predisposing agents or to the triggering insults results, by itself, in no alterations in renal function or histology. However, when animals were subjected sequentially to both the predisposing agent and the triggering insult, an overt, intrinsic AKI ensued, as evidenced functionally by increased plasma creatinine and urea concentrations and decreased creatinine clearance; and histologically by ATN, with widespread tubular dilation and degeneration, massive cell death, tubular casts and inflammatory cell infiltration. Interestingly, the increase in the urinary transferrin excretion was higher in animals predisposed to AKI following most subnephrotoxic

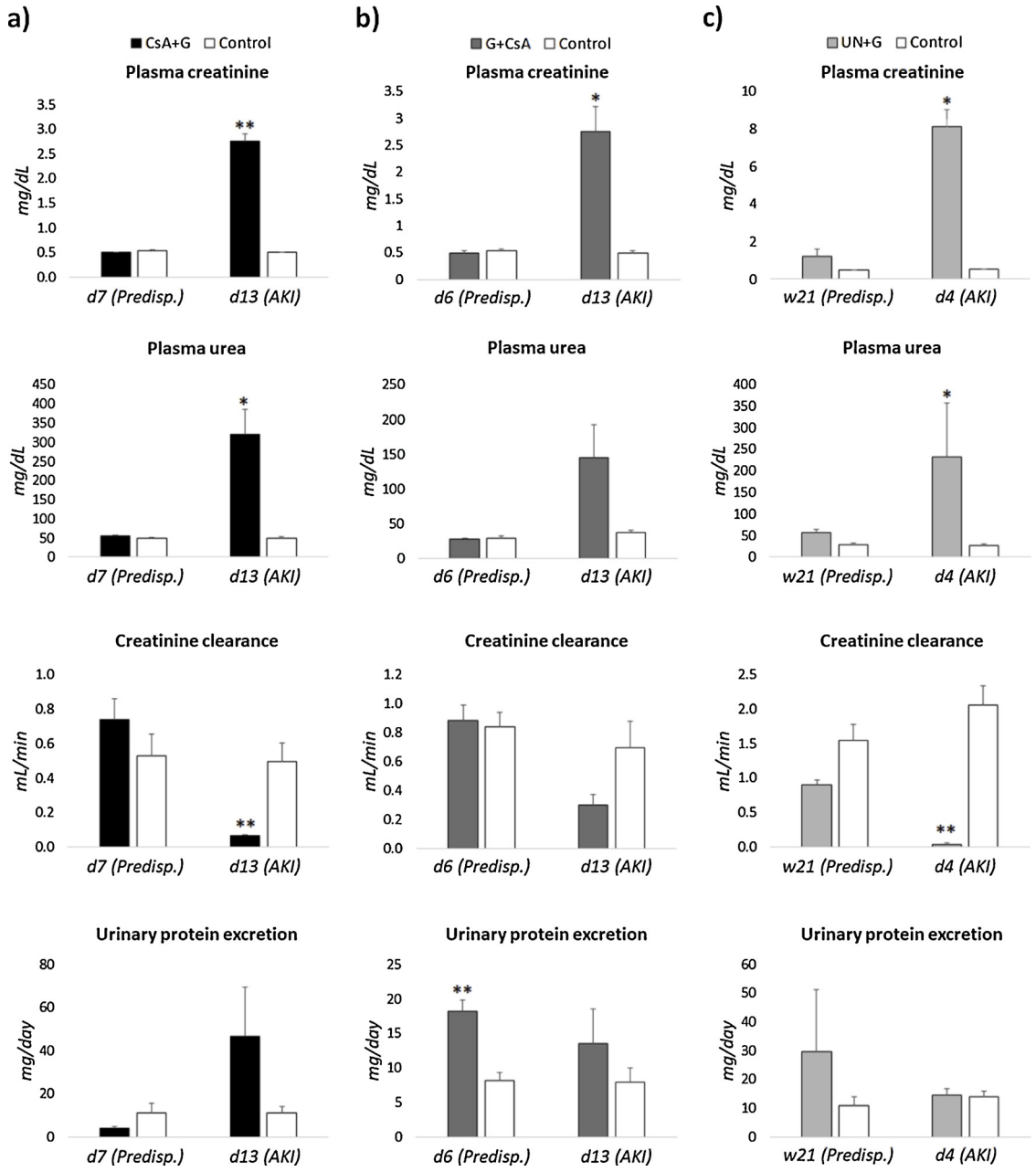

Fig. 3. Plasma creatinine and urea levels, creatinine clearance and urinary protein excretion in different rat models of predisposition to AKI. Values are expressed as the mean ± SEM. *, $p < 0.05$; **, $p < 0.01$ vs. Control group. Predisp., predisposition; d, day; w, week; CsA, cyclosporine; G, gentamicin; UN, uranyl nitrate.

regimes (i.e. cisplatin, gentamicin and uranyl nitrate), but not following cyclosporine, when compared to controls receiving the vehicle instead (Fig. 5). Of note, urinary transferrin was elevated following administration of cisplatin dosages (i.e. 2.5 and 3.0 mg/kg) that predispose to subsequent triggers of AKI, but not following the non-predisposing dosage (i.e. 2.0 mg/kg) (see Fig. 5 for transferrin values, and Fig. 2 for evidence of predisposition). Overall, these data strongly link increased urinary transferrin to acquired predisposition to AKI. Additionally, urinary transferrin predicted the severity of the subsequent AKI developed upon administration of the triggering agent. Specifically, the increment in urinary transferrin excretion induced by the predisposing agent statistically correlates with the severity of renal damage caused by the triggering insult (Fig. 6). Indeed, the higher the level of transferrin prior to the administration of the triggering insult, the higher the level of plasma creatinine and urea reached. Finally, the predictive capacity of two markers of subtle and subclinical renal damage, i.e. as urinary NAG and NGAL, was also tested. Table 2 shows that neither marker shows any positive correlation with the subsequent renal damage.

3.2. Increased urinary excretion of transferrin results from decreased tubular uptake

In order to unravel the origin and mechanism of the increased levels of urinary transferrin found in rats predisposed to AKI and, specifically, whether it was the result of a subclinical tubular alteration that would corroborate our hypothesis, we used the subclinical cisplatin predisposition model. The right kidney was ligated and the left kidney was perfused *in situ* with a protein-free solution (i.e. Krebs-dextran). Urine was collected prior to and during Krebs perfusion in rats treated with a subnephrotoxic dose (3 mg/kg) of cisplatin. Fig. 7a shows that transferrin disappears from the urine of cisplatin-predisposed rats when the kidney is perfused with Krebs solution. This supports the idea that

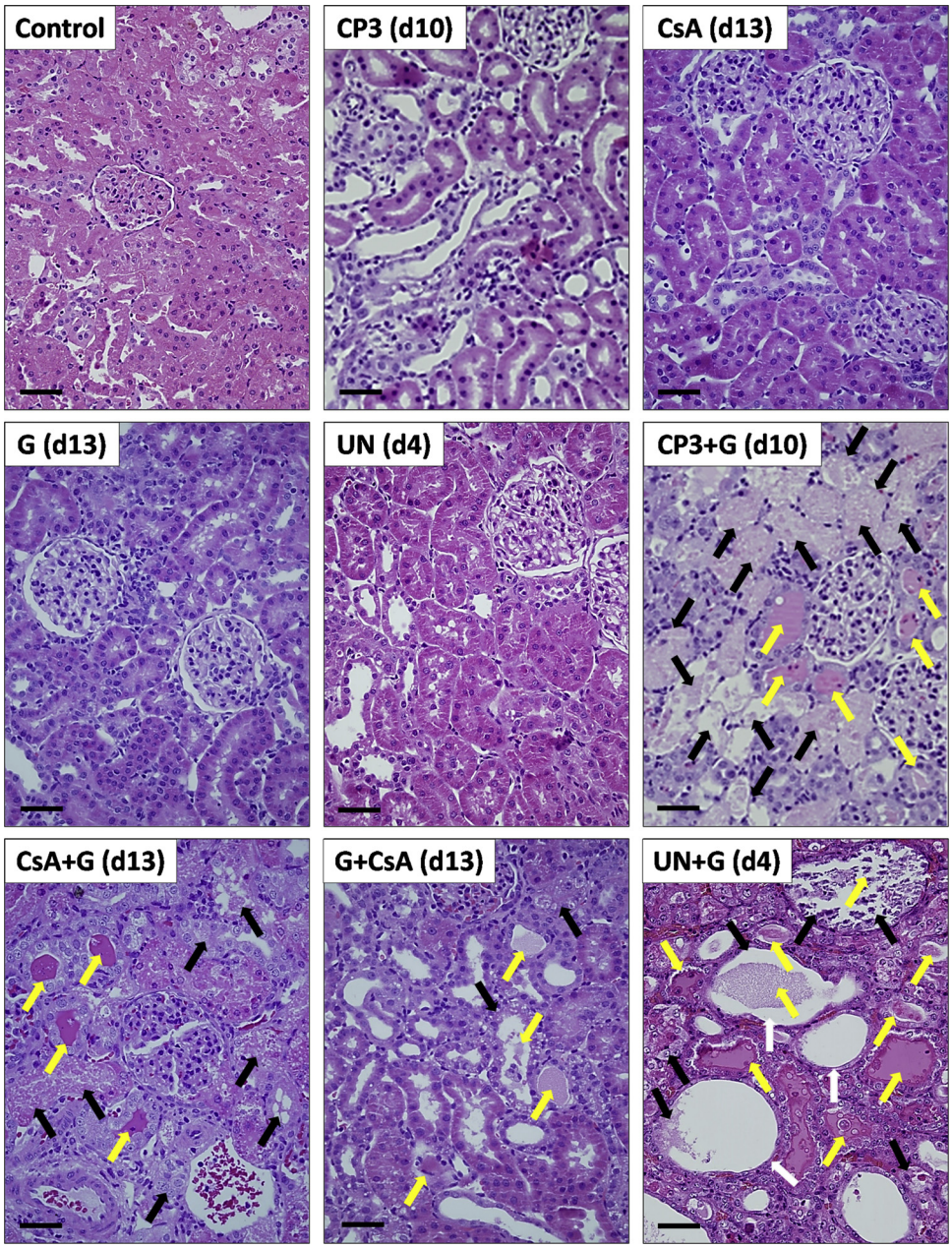

Fig. 4. Representative images of renal histology in rat models of predisposition to AKI. Scale bar: 50 μm. CP: cisplatin; CsA: cyclosporine; G: gentamicin; UN: uranyl nitrate. Whereas individual (single) treatments cause no signs of gross tissue damage, all combinations of predisposing + triggering factors show an evident tubular necrosis characterized by tubule loss and obstruction. Black arrows indicate areas of tubular necrosis with massive de-epithelialized tubules, and tissue debris. Yellow arrows show areas of severe tubular obstruction (with hyaline material). White arrows point at dilated tubules (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

urinary transferrin comes from the blood and not from renal structures. Furthermore, inhibition of proximal tubule transport with maleate in control rats resulted in a sustained increase in urinary transferrin (Fig. 7b). These results support a mechanism of transferrin trafficking to urine in rats predisposed to AKI, whereby a subclinical defect in its tubular handling (i.e. probably decreased proximal tubule reabsorption) results in increased urinary transferrin excretion.

Our results from *in situ* perfused kidneys, and from the maleate model suggest that the increased levels of urinary transferrin found in rats predisposed to AKI result from an altered tubular reabsorption.

3.3. Urinary transferrin pre-emptively identifies oncological patients subsequently undergoing platinum-based therapy nephrotoxicity and cardiac patients subsequently suffering contrast-induced nephropathy

The clinical utility of transferrin as a pre-emptive marker of predisposition to, or risk of, kidney damage was evaluated in two groups of patients: (i) oncological patients who were to be treated with cisplatin or carboplatin; (ii) patients with heart disease who were to be subject to a procedure involving the administration of an iodinated contrast medium. In both cases transferrin was measured before the administration of the cytostatic or contrast medium, with the objective of studying whether its urinary levels were predictive of subsequent renal damage. Patients were then monitored for evidence of newly developed nephrotoxicity following treatment. Before treatment, patients are expected to have a range of baseline, individual risk values depending on an undetermined mixture of predisposing conditions impossible to identify (resulting from their individual exposure to predisposing agents, lifestyle, comorbidities, etc.) and, hitherto impossible to detect and quantify by existing diagnostic technology.

3.3.1. Study 1: oncology

70 patients initially volunteered, but 35 were excluded (27 died or withdrew consent; and 8 had abnormal baseline values for two of the four parameters of nephrotoxicity, including plasma creatinine). Finally, 35 patients completed the study. Of them, 18 were considered Cases since they developed new evidence of nephrotoxicity. The other 17 patients showed no evidence of nephrotoxicity, and were labelled as

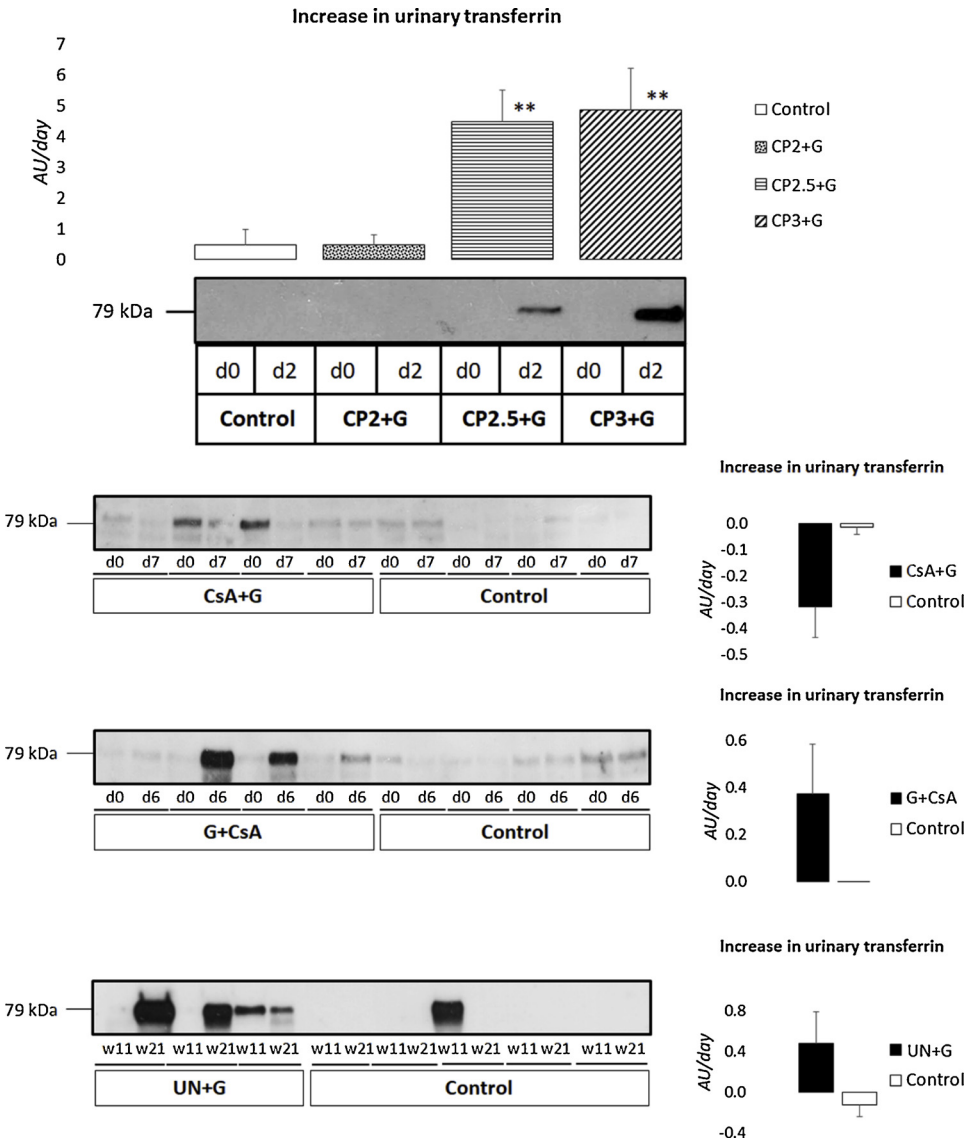

Fig. 5. Increase in urinary transferrin excretion during the predisposition stage in different animal models: Representative Western blot images of urinary transferrin and the corresponding densitometry quantification. "Increase in urinary transferrin" reflects the difference between the selected time point and baseline values (i.e. day zero or week 11, depending on the model). Both time points preceded the administration of the second (triggering) insult (i.e. subnephrotoxic gentamicin or CsA). Control animals received saline or vehicle. Values are expressed as the mean ± SEM. **, $p < 0.01$ vs. Control group. AU, arbitrary units; d, day; w, week; CP, cisplatin; CsA, cyclosporine; G, gentamicin; UN, uranyl nitrate.

Controls. Their initial anthropometric and pharmacological data are presented in Table 1.

3.3.2. Study 2: cardiology

Of the 172 patients initially included, 19 voluntarily left the study. Finally, 153 patients completed the study. Out of them, 31 were considered Cases, because they developed CIN. The other 122 patients did not develop CIN and, therefore, they were considered Controls. Their initial anthropometric and pharmacological data are presented in Table 1.

As shown in Fig. 8, when measured before the initiation of platinum-based therapy, the urinary levels of transferrin were significantly higher in Cases, i.e., in patients who subsequently developed evidence of nephrotoxicity, than in Controls who did not. ROC curves were obtained for the capacity of urinary transferrin to pre-emptively discriminate patients subsequently becoming Cases and Controls (a). Similarly, when measured before the administration of the iodinated contrast medium, urinary levels of transferrrin were significantly higher in Cases, i.e., in patients who subsequently developed evidence of CIN, than in Controls who did not. Also, ROC curves were obtained for the capacity of urinary transferrin to pre-emptively discriminate patients subsequently becoming Cases and Controls (b). Congruently with the observations in animal models, sensitive biomarkers of subclinical renal damage (NAG and NGAL) have no significant predictive capacity in either clinical setting (Fig. 9). Mean values are not statistically different between Cases and Controls, and ROC curves show much lower predictive value than transferrin.

4. Discussion

Overall, our data indicate that an increased urinary excretion of transferrin is strongly associated to the acquired predisposition to AKI induced by subnephrotoxic exposure to different nephrotoxins in rats. Also that in predisposed animals, urinary transferrin tightly correlates with the severity of the subsequent renal dysfunction triggered by exposure to a second insult that is not toxic to non-predisposed rats. And finally, that urinary transferrin pre-emptively identifies a fraction of oncological patients that will develop evidence of nephrotoxicity following treatment with cisplatin or carboplatin, and a fraction of cardiology patients who will subsequently suffer CIN.

From the existing animal models of acquired predisposition to AKI [19–21] no common mechanism and biomarker of predisposition had been previously identified, but only model-specific biomarkers. Interestingly, our present results show that transferrin is a biomarker of several animal models of predisposition resulting from exposure to diverse stimuli, and also of an etiopathologically heterogeneous, clinical

Increase in transferrin excretion	Correlation with plasma creatinine		Correlation with plasma urea	
	R Pearson	p-value	R Pearson	p-value
Cisplatin model	0.68	0.001**	0.53	0.02*
Cyclosporine model	-0.78	0.02*	-0.63	0.09
Gentamicin model	0.88	0.004**	0.99	$4{\cdot}10^{-6}$***
Uranyl nitrate model	0.73	0.04*	0.77	0.03*

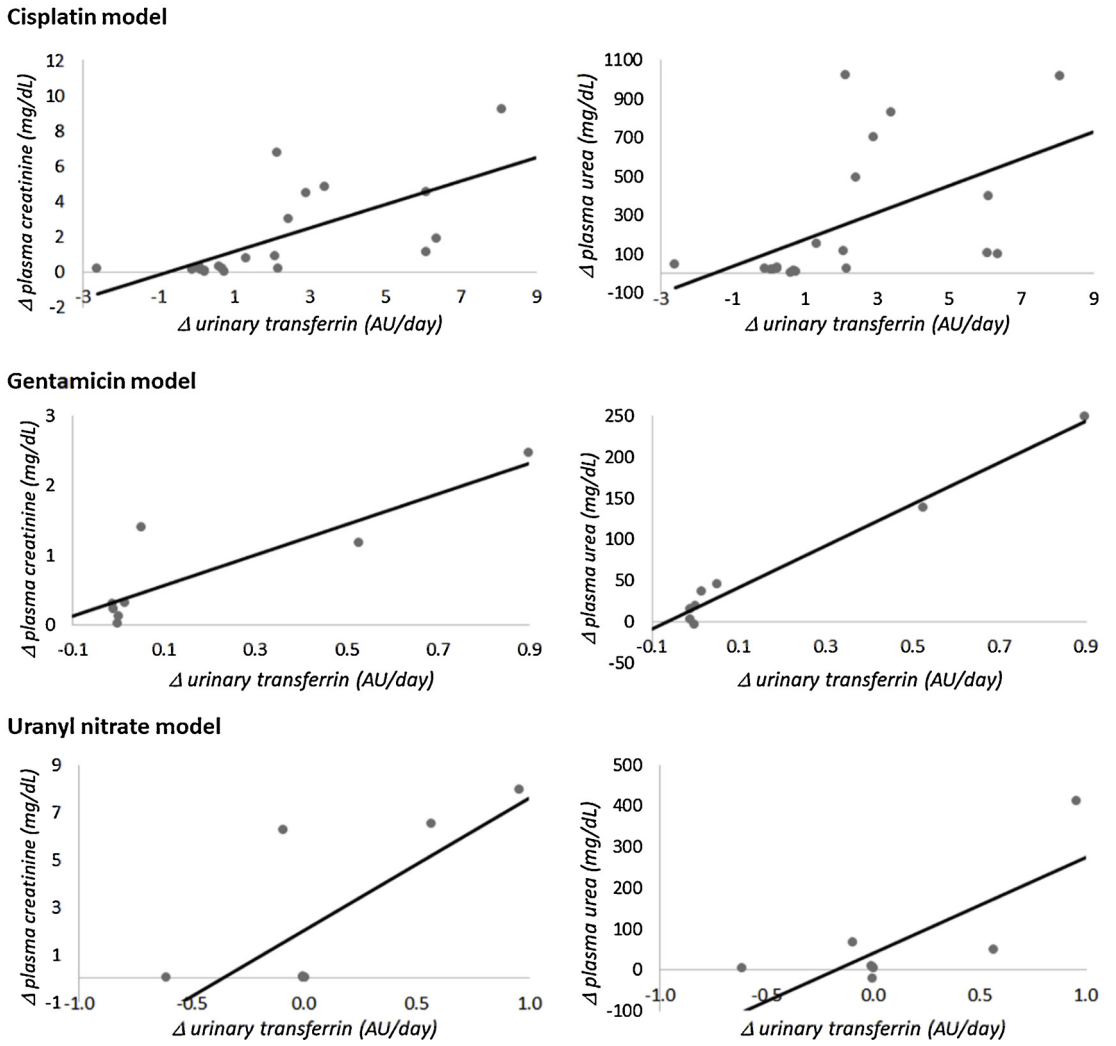

Fig. 6. Correlation between urinary transferrin excretion during the predisposition stage and the increase in plasma creatinine and urea during the AKI stage. AU: arbitrary units; R Pearson: Pearson's correlation coefficient.

condition predisposing to platinum chemotherapy nephrotoxicity. These facts suggest that a common mechanism may underlie many forms of predisposition to AKI and, congruently, a common biomarker could be characterized. In this sense, our study also sheds some light on the underlying mechanisms of predisposition to AKI identified by elevated urinary transferrin. Transferrin is a 79-kDa plasma protein produced in the liver, shed to the circulation where it binds one or two Fe^{3+} atoms, and therefrom endocytosed by many cell types for Fe delivery, through specific plasma membrane receptors (TfR1 and TfR2) [46,47]. Transferrin is known to be filtered to a certain extent through the glomerular filtration barrier, and mostly reabsorbed in the proximal tubule [48]. The luminal membrane of proximal tubule epithelial cells contains both TfRs and the megalin-cubilin complex, another transport system known to internalize many molecules including transferrin [48–50]. Our results from *in situ* perfused kidneys, and from the maleate model suggest that the increased levels of urinary transferrin found in rats predisposed to AKI result from an altered tubular reabsorption. And these results also indicate that subclinical tubular dysfunction is associated, and probably etiologically related, to a common mechanism of predisposition to AKI underlying several etiologically distinct animal models. Congruently with this, transferrin identifies the predisposition caused by nephrotoxins known to accumulate in and affect the proximal tubules, i.e. gentamicin [51,52], cisplatin [53] and uranyl nitrate [54]. In contrast, the predisposition induced by cyclosporine A is not detected by transferrin, coinciding with a different mechanism. In fact, cyclosporine nephrotoxicity is thought to result from renal vasoconstriction and altered renal hemodynamics [55].

In agreement with these animal studies, the level of urinary transferrin identifies a fraction of patients predisposed to the nephrotoxicity of platinum-based oncological treatments and iodinated contrast media

Table 2
Correlation between urinary NAG or NGAL excretion during the predisposition stage and the increase in plasma creatinine and urea during the AKI stage. NAG: N-acetyl-β-D-glucosaminidase; NGAL: neutrophil gelatinase-associated lipocalin; R Pearson: Pearson's correlation coefficient.

Increase in NAG excretion	Correlation with plasma creatinine		Correlation with plasma urea	
	R Pearson	p-value	R Pearson	p-value
Cisplatin model	−0.06	0.81	−0.14	0.56
Cyclosporine model	−0.05	0.90	−0.06	0.89
Gentamicin model	0.29	0.48	0.48	0.23
Uranyl nitrate model	0.32	0.45	0.75	0.04*
Increase in NGAL excretion				
Cisplatin model	−0.10	0.69	−0.15	0.53
Cyclosporine model	0.05	0.91	−0.12	0.78
Gentamicin model	0.53	0.18	0.55	0.16
Uranyl nitrate model	0.57	0.14	0.80	0.017*

* statistically significant difference.

in a non-selected and heterogeneous population. In both cases, thus, before exposure to the potentially nephrotoxic agent, patients had individual levels of risk of renal damage produced by a specific but heterogeneous mix of undetermined factors, unknown to the specialist. As shown in Fig. 8, according to the ROC curves, the predictive efficacy of urinary transferrin is higher in the oncology study than in the cardiology study. Following our own argument, this difference is congruent with the different mechanism of nephrotoxicity of platinated antineoplastics and iodinated contrast media. Whereas antineoplastics mainly alter the renal tubules [33], contrasts have both vascular and hemodynamic effects, and also tubular toxicity [56,57]. In the case of antineoplastics, transferrin seems to detect more patients because, logically, subclinical tubular alterations are more likely to predispose to further tubular damage. Whereas in the case of iodinated contrasts, CIN may have a more pronounced tubular phenotype in some patients (those identified by transferrin); and a bolder vascular and hemodynamic phenotype others (i.e. those not identified by transferrin), who are less expected to be predisposed (or to a lower extent) by subclinical tubular alterations (i.e. those inducing the increase in urinary transferrin).

Altogether, our preclinical and clinical results suggest that elevated urinary transferrin may be a biomarker resulting from the core of a common, not yet sufficiently characterized, mechanism of predisposition to renal damage involving some degree of proximal tubule

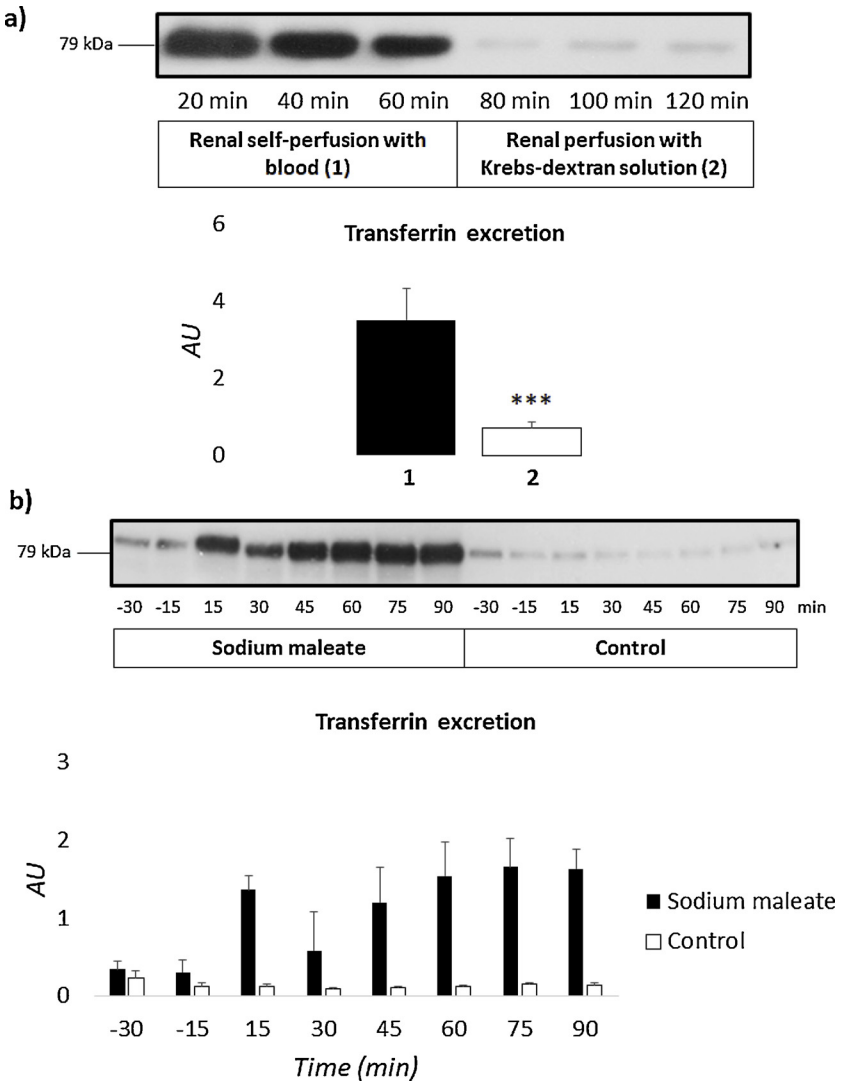

Fig. 7. Representative images of Western blot analysis and the corresponding densitometry quantification of urinary transferrin excretion, a) during the phase of auto-perfusion with blood and during the Krebs-dextran perfusion phase; and b) before (-30 and -15 min) and after (15 min) administration of sodium maleate or saline vehicle. Values are expressed as the mean ± SEM. ***, $p < 0.001$ vs. 1. AU, arbitrary units.

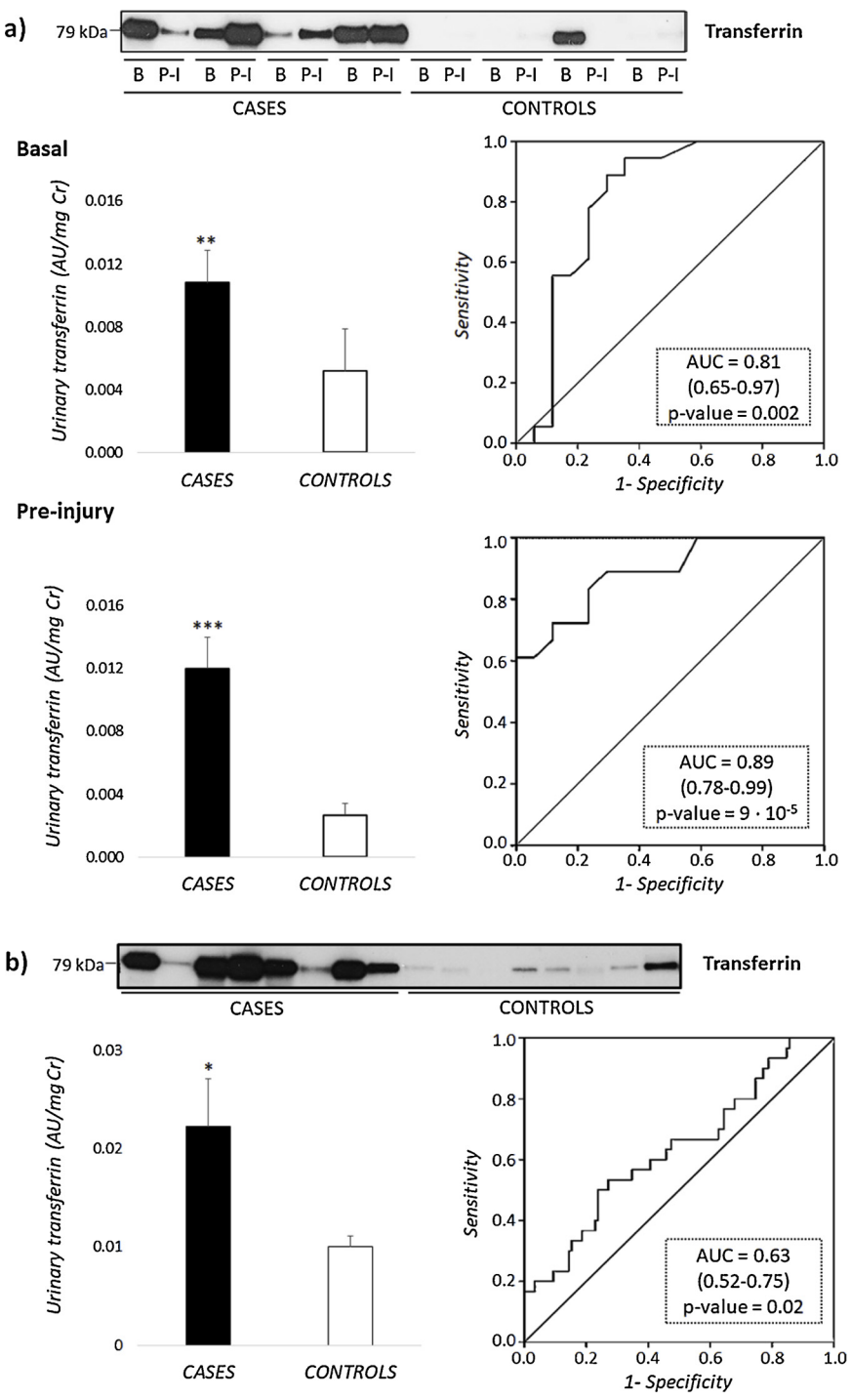

Fig. 8. a) Basal and pre-injury urinary levels of transferrin in samples from Cases and Controls patients in a) oncology patients treated with platinated antineoplastics (Study 1); and b) basal urinary levels of transferrin in samples from Cases and Controls in cardiology patients subject to iodinated contrast media (Study 2). Representative images of Western blot analysis and its corresponding densitometry quantification; and evaluation of diagnostic performance of urinary transferrin through ROC curves and area under the curves values (95 % CI). Data are expressed as the mean ± SEM. AU: arbitrary units; AUC, area under the curve; B, basal; Cr, creatinine; P—I, pre-injury. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$ vs. Controls group.

subclinical dysfunction, which merits further investigation. We speculate that a renal damage-predisposing agent activating this mechanism may totally or partially exhaust the proximal tubule functional reserve. As a result, when in these circumstances the kidneys are challenged by a level of exposure to the triggering insult that would have no consequences in non-predisposed kidneys, proximal tubules would be incompetent to fully reabsorb the ultrafiltrate load, leading to the activation of the tubuloglomerular feedback mechanism and to a reduction in GFR, and compromising hydroelectrolytic balance. With this knowledge, a personalized approach to therapy may be implemented by selectively monitoring more intensely those patients at high risk of nephrotoxicity, by individually adjusting treatment and posology, and by implementing preventive measures to optimize kidney outcomes. These results provide a step forward and the proof-of-concept for the clinical application of a conceptually new strategy to manage drug nephrotoxicity and AKI in a truly preventive and personalized manner, hitherto impossible to detect and thus impossible to implement in the clinical practice. This approach may help to minimize acute nephrotoxicity and its long-term sequelae, such as the need for chronic dialysis [22].

This study poses a base for a larger, multicenter study, as sample size is a limitation of this study. It also invites to explore the capacity of urinary transferrin to pre-emptively detect the risk of kidney injury in other clinical circumstances in which a potentially kidney-injuring insult, treatment or clinical procedure, has a known timeline, and allows to preventively personalize risk stratification and risk-reducing

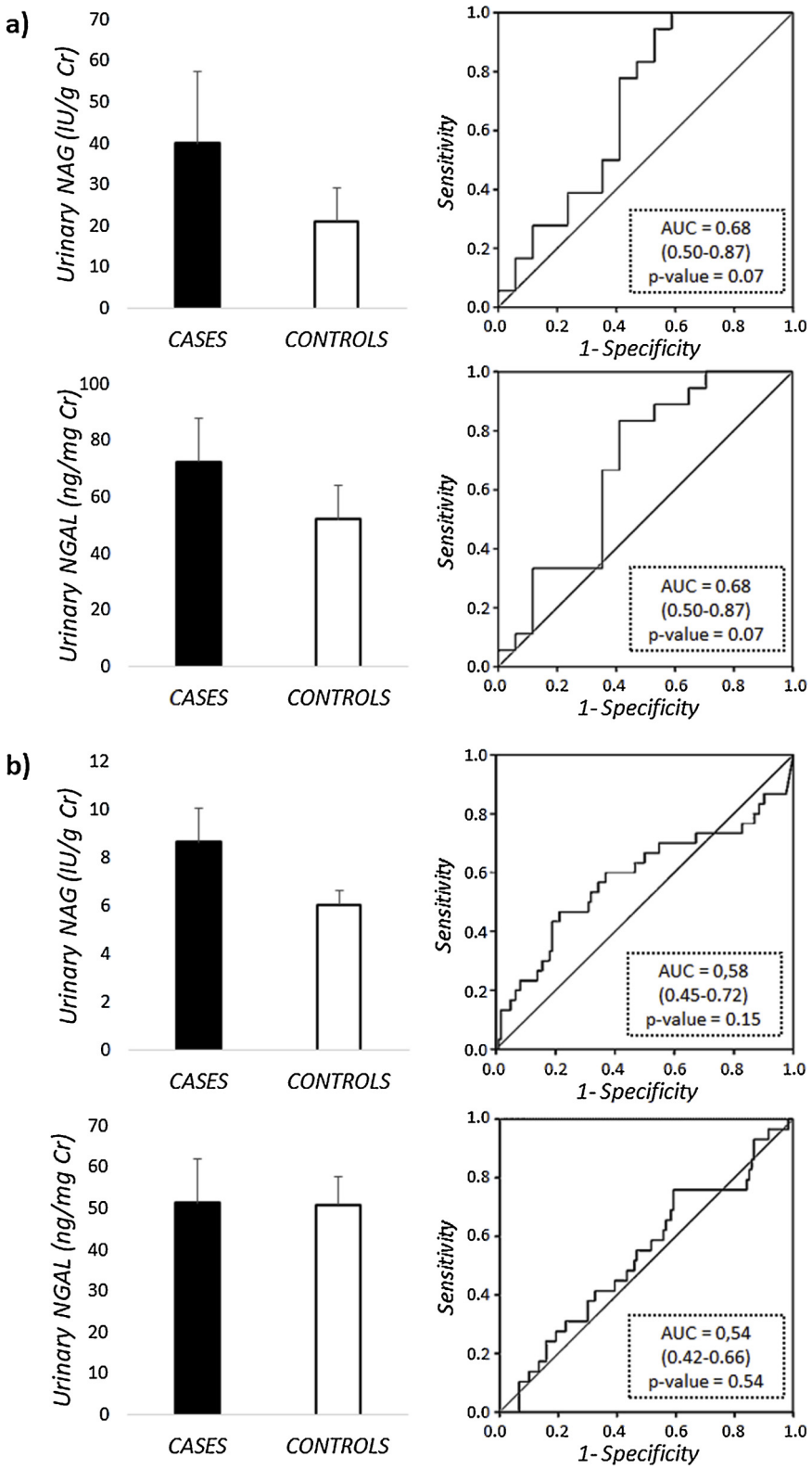

Fig. 9. Urinary levels of NAG and NGAL in basal samples from Case and Control patients, and evaluation of diagnostic performance of urinary transferrin through ROC curves in a) oncology patients treated with platinated antineoplastics (Study 1); and b) cardiology patients subject to iodinated contrast media (Study 2). Area under the curve (AUC) values (95 % CI). In left panels, data are expressed as the mean ± SEM. Cr, creatinine; NAG, N-acetyl-β-D-glucosaminidase; NGAL, neutrophil gelatinase-associated lipocalin.

strategies. In these situations, anticipation of AKI risk at the individual level, may help to avoid potentially harmful treatments or procedures in high risk patients; whereas to use them with confidence (or even at higher intensity, if necessary) in low risk patients. Finally, urinary transferrin might also have a diagnostic role in the community for the monitoring of risk of AKI in the general population through routine outpatient analysis and in the context of family medicine.

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Declaration of Competing Interest

The authors declare that there is no conflict of interest regarding the publication of this article.

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