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SHORT COMMUNICATION

Urinary extracellular vesicles as a monitoring tool for renal damage in patients not meeting criteria for chronic kidney disease

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Abstract

Background: Current definition of chronic kidney disease (CKD) identifies only advanced stages, but effective management demands early detection. Urinary albumin-to-creatinine ratio (ACR) 30 mg/g is a cut-off point for CKD clinical diagnosis. Patients with lower values (normoalbuminuria) and eGFR > 60 mL/min/1.73 m^2 are considered at no increased cardiorenal risk. However, higher incidence of renal function decline and cardiovascular events have been shown within the normoalbuminuria range. Novel subclinical indicators may help to identify higher-risk patients. Urinary extracellular vesicles (uEVs) are sentinels of renal function non-invasively. Here we aimed to approach the early assessment of cardiorenal risk by investigating the protein cargo of uEVs.

Methods: Hypertensive patients were classified in control group (C) with ACR < 10 mg/g, and high-normal group (HN) with ACR 10–30 mg/g. Isolated uEVs were characterized by western blotting and electron microscopy and the protein cargo

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was analyzed by untargeted proteomics (LC-MS/MS) in a first discovery cohort. Protein confirmation was performed in a different cohort by ExoView. Immunohistochemistry of human kidney biopsies was also performed to evaluate the potential of uEVs to reflect renal damage.

Results: HN albuminuria does not affect the uEVs concentration, size, or tetraspanin profile. Among >6200 uEVs proteins identified, 43 define a panel significantly altered in HN patients without variation in urine, mostly annotated in the tubule (39 out of 43). The tubular transporter long-chain fatty acid transport protein 2 (SLC27A2) and the apical membrane protein amnionless (AMN) confirmed their alteration in HN patients evidencing impaired tubular reabsorption. SLC27A2 showed tubular expression and significantly reduced levels in patients with diagnostic criteria for CKD.

Conclusions: Alterations in the EV-mediated molecular profile are evident before pathological ACR levels are reached. Direct quantitation of SLC27A2 and AMN in uEVs helps identifying normoalbuminuric subjects with higher cardiorenal risk in early monitoring of CKD.

KEYWORDS
albuminuria, AMN, cardiovascular risk, chronic kidney disease, ExoView, extracellular vesicles, FATP2, proteomics, SLC27A2

1 | INTRODUCTION

Chronic kidney disease (CKD) is expected to be the leading cause of death worldwide by 2040 with unmet needs for early diagnosis and treatment. The current definition of CKD identifies only advanced stages of the disease, while its early detection is crucial for effective management (Ortiz, 2022; Ruilope et al., 2023). One of the main criteria to diagnose and categorize CKD is the presence of moderately increased albuminuria, with a cutoff value ≥30 mg/g urinary albumin-to-creatinine ratio (ACR). Moderately increased albuminuria is an established risk factor of cardiovascular morbimortality and renal disease, having an additive effect on the risk of fatal long-term prognosis for renal disease at any stage of estimated glomerular filtration rate (eGFR) (Polkinghorne, 2014). Despite subjects with ACR < 30 mg/g (normoalbuminuria) and estimated eGFR > 60 mL/min/1.73 m^2 are considered at no increased cardiorenal risk in clinical practice, cardiovascular risk and renal function decline associate with urinary albumin concentration further below the stablished cut-off for risk, that is, within the normoalbuminuria range (Hallan et al., 2012; Kang et al., 2022; Melsom et al., 2018). Cardiovascular events (Blecker et al., 2011; Ruggenenti et al., 2012), increased incidence of CKD (Okubo et al., 2020) and a faster decline of eGFR has been reported for normoalbuminuric subjects within the high-normal range (ACR = 10–30 mg/g) (Melsom et al., 2018). By being below the threshold levels considered pathological, no therapeutic action is envisaged supporting the "blind-spot" concept in CKD diagnosis, that is, when kidney injury is present but is undetectable by current diagnostic criteria and no intervention is made before renal and cardiovascular damage occurs (Ruilope et al., 2023). The solution lies in implementing the measurement of ACR with new biological indicators that also indicate potential therapeutic mechanisms of action in the early and subclinical stages of albuminuria progression (Ruilope et al., 2023; Sehestedt et al., 2010). In this way, the identification of patients at apparent no cardiorenal risk would allow early intervention to preserve kidney function and prevent cardiovascular events.

Extracellular vesicles (EVs) participate in cellular communication within the kidney functional units, including glomerular–tubular crosstalk (Martinez-Arroyo et al., 2021; Munkonda et al., 2018). In particular, urinary EVs (uEVs) reliably reflect protein changes occurring in the kidney (van Heugten et al., 2022; Wu et al., 2021) so that renal damage may be reflected in urine through monitoring of changes in uEVs (Burrello et al., 2023; Zubiri et al., 2015). We have previously shown proteins and metabolites variations in urine from patients within the normoalbuminuria range, pointing to altered tubular reabsorption (Santiago-Hernandez et al., 2021; Santiago-Hernandez et al., 2021). Here we aimed to investigate whether the protein cargo of uEVs also allow stratifying the normoalbuminuria range according to cardiorenal risk and reflect molecular alterations associated to kidney damage in early and subclinical CKD.

2 | METHODS

2.1 | Patient recruitment and urine sample collection

A total of 32 patients with hypertension and ACR values within the normoalbuminuria range were recruited from the Hypertension Unit of the Hospital 12 de Octubre, Madrid, Spain. Patients with secondary hypertension or resistant hypertension were excluded. Blood pressure was measured at the office with a validated semiautomatic oscillometric device, after 5-min resting in a sitting position. BP values were estimated as the mean of three readings. Patients were classified in two groups based on their ACR, according to cardiorenal risk stratification by KDIGO guidelines in the normoalbuminuria range (KDIGO 2012, 2012): a control group (C), which included patients with ACR < 10 mg/g (no progression to renal disease is expected) and a high-normal group (HN) with an ACR 10–30 mg/g (susceptible of progression to pathological albuminuria and CKD). The study was approved by the local Ethics Committee (PIC139-2016) and adhered to the principles of the Declaration of Helsinki. Informed consent was obtained from all patients indicating that participation in the study was not prejudicial in any way to treatments and possessed no risk.

2.2 | uEV isolation and characterization by western blotting and electron microscopy

The study was performed in accordance with the minimal information for studies of extracellular vesicles (MISEV) guidelines for the study of urinary extracellular vesicles (uEVs) whenever possible (Théry et al., 2018). First morning urine samples were collected in sterile containers and transported to the laboratory within a period no longer than 3 h. A protease inhibitor cocktail (Sigma P8340) was added and samples were stored at −80°C until analysis. uEVs were isolated from urine by ultracentrifugation, as described (Gonzalez-Calero et al., 2017; Zubiri et al., 2013, 2014). Briefly, urine samples were defrosted at 37°C, vortexed and centrifuged (17,000 *g*, 10 min, 4°C), and the supernatant was collected. The pellet was treated with 200 mg/mL dithiothreitol in PBS (150 mM NaCl, 12.5 mM Na_2HPO_4, 11.5 mM NaH_2PO_4, in ddH_2O, pH 7.2) and heated at 37°C for 10 min to reduce Tamm–Horsfall protein networks and to release trapped uEVs. Following a second centrifugation round (17,000 *g*, 10 min, 4°C), the supernatant was collected, added to the first supernatant and then ultracentrifuged (175,000 *g*, 70 min, 4°C) to pellet the uEVs. Size and morphology were determined by electron microscopy by suspending the uEV pellet in 4% paraformaldehyde/PBS. For western blotting analysis, the following primary antibodies were used: Alix (1:500, Santa Cruz; ref: SC-53540), TSG-101 (1:500, Abcam; ref: ab83) and Syntenin-1 (1:500, Novus Biologicals; ref: H00006386-B01P). A rabbit anti-mouse-HRP (1:2500, NORDIC MUbio) was used as a secondary antibody.

2.3 | Untargeted proteomics of isolated uEVs

In a first discovery phase, quantitative proteomics was performed by isobaric labelling and liquid chromatography coupled to mass spectrometry (LC-MS/MS). Differential protein quantitation was performed by multiplexed tandem mass tag (TMT) isobaric labelling in a patients' cohort including 10 subjects (5C and 5HN). uEV pellets were solubilized in lysis buffer (7M urea, 2M urea, 4% CHAPS, 30 mM Tris-HCl) and 30 μg of total protein per sample were digested with modified trypsin (protein:trypsin ratio, 40:1) using the filter-aided sample preparation method with slight modifications (Wiśniewski et al., 2009). The resulting peptides were acidified with trifluoroacetic acid and desalted on OASIS HLB columns (Waters). Peptides were solubilized in 200 mM TEAB buffer, quantified using a direct detect system (Millipore), and labelled with TMT isobaric labelling reagent (TMT-10plex™ Kit, Fisher Scientific). TMT-labelled peptides were desalted on OASIS HLB columns and an aliquot was fractioned on a C18 reversed-phase column (High pH Fractionation Kit; Thermo Scientific) into six fractions and analysed by LC-MS/MS on a Q Exactive HF Hybrid Quadrupole Orbitrap Mass Spectrometer (Thermo Scientific). Peptides were loaded onto a pre-column (PepMap100 C18 LC 75 μm ID × 5 cm, 2 μm, Thermo Scientific) and separated on-line on a NanoViper PepMap100 C18 LC analytical column (75 μm ID × 50 cm, Thermo Scientific) in a continuous acetonitrile gradient. An enhanced Fourier transform-resolution spectrum (resolution = 70,000) followed by MS/MS spectra acquisition from the most intense parent ions were analysed along the chromatographic run. Dynamic exclusion was set at 40 s. For peptide identification, fragmentation spectra were analysed with Proteome Discovery (2.1.0.81 version; Thermo Scientific) and SEQUEST-HT (Thermo Scientific). The Uniprot database (SwissProt and trembl proteome; target/decoy) was used for protein assignment (download on 2021/05; 197,508 sequences). Peptide identification was performed using the probability ratio method (Martínez-Bartolomé et al., 2008). The false discovery rate (FDR) was calculated using inverted databases and the refined method using a cut-off value of 1% FDR (Navarro & Vázquez, 2009), with an additional filtering for precursor mass tolerance of 15 ppm (Bonzon-Kulichenko et al., 2015). Statistical analysis was performed using the weighted spectrum, peptide and protein (WSPP) model, as described (Navarro et al., 2014). In this model, protein log2-ratios are expressed as standardized variables, consisting of units of standard

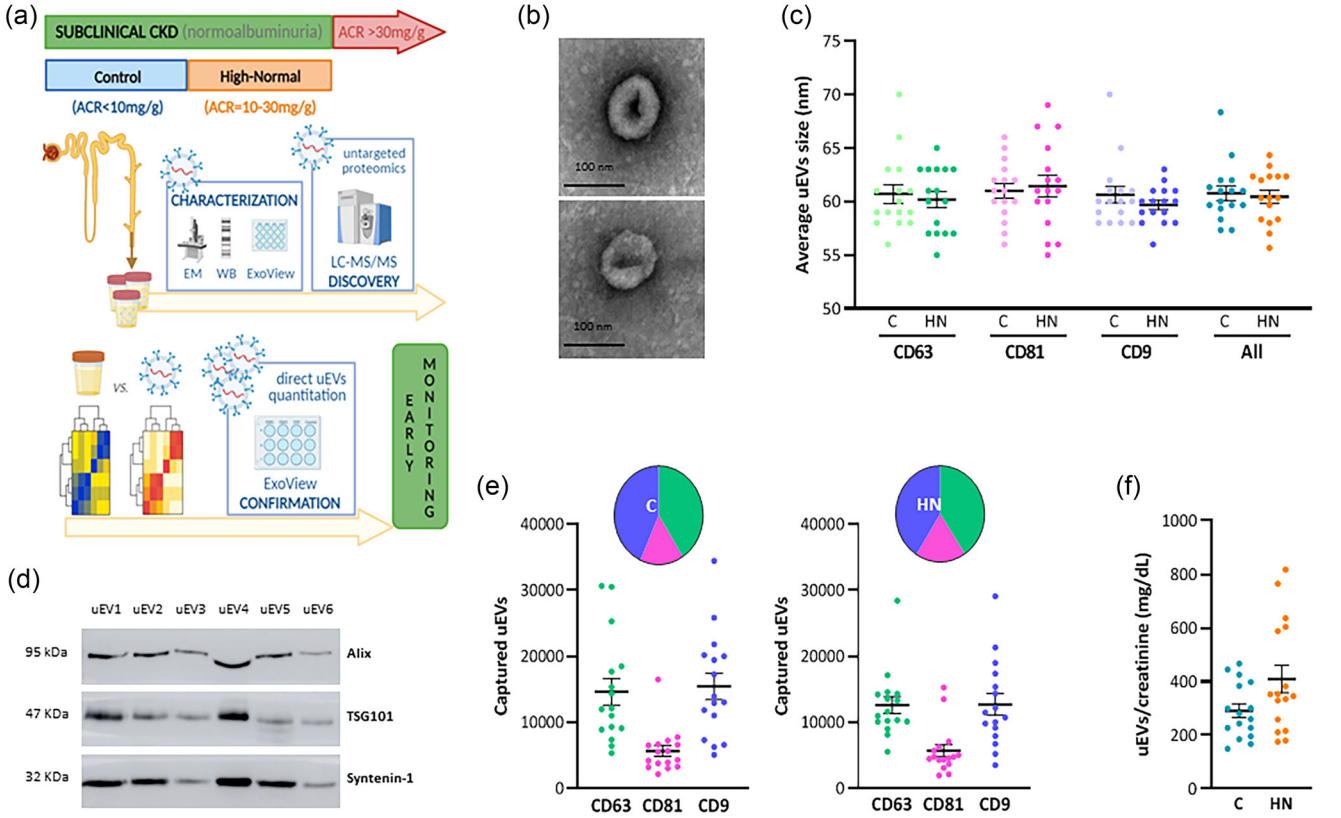

FIGURE 1 Subclinical albuminuria does not affect the concentration, size or tetraspanin profile of uEVs. (a) Study workflow of uEV analysis in controls (C,) and patients with high-normal albuminuria (HN). uEVs were characterized by electron microscopy (b), ExoView (c) and western blotting (d). Tetraspanin profile of uEVs is depicted (e) and the total number of quantified uEVs normalized by urinary creatinine (f).

deviation according to their estimated variances (Zq values). Changes in protein abundance between groups were calculated by comparing the Zq median values (ΔZq). Significance was established at $p \leq 0.05$.

2.4 | Comparative analysis between uEV and urine proteomes

Protein variations identified in uEVs between the C and HN clinical groups were compared with our previous data on urinary proteins from C and HN patients (Santiago-Hernandez et al., 2021). For comparison, individual urinary protein variations are shown in an open repository as supplementary material (named HJH_2021_06_14_ALVAREZLLAMAS_JH-D-21-00331_SDC2.xlsx) included in Santiago-Hernandez et al. (2021). Proteins identified both in urine and in uEVs but showing differential variation between clinical groups only in uEVs were classified according to their annotated expression in the glomerulus and/or the tubule based on the human protein atlas and the extant literature.

2.5 | Systems biology analysis and coordinated protein alterations

Coordinated protein changes and functional categories were analysed using the systems biology triangle (SBT) algorithm (García-Marqués et al., 2016), which identifies changes in biological processes far beyond individual protein responses and assigns a Z score to each category (Zc). Differences in biological processes were evaluated by measuring the difference in median Zc values (ΔZc) between the clinical groups. Significance criteria were ≥5 proteins implicated and $p \leq 0.05$. Functional categories were compared with those previously obtained from urine data shown in Santiago-Hernandez et al. (2021), Figure 1. Protein interaction analysis was also performed using STRING 11.5 software (highest confidence, k-means clustering).

2.6 | Multiparametric analysis of uEVs using the ExoView platform: Tetraspanin profiling and protein cargo targeted quantitation

In a second confirmation stage, uEVs from 32 patients were directly analysed in 32 chips (16C and 16HN) without prior isolation from urine, using the single particle interferometric reflectance imaging sensor (SP-IRIS) Platform/ExoView R200 analyser (Unchained labs). The commercial human standard tetraspanin kit (EV-TETRA-C) consisting of anti-CD63, anti-CD81, and anti-CD9 capture antibodies was used. uEV characterization was performed according to size (nm), tetraspanin profile and number of uEVs. The concentration and vesicle localization of the two tubular proteins long-chain fatty acid transport protein 2 (SLC27A2) and protein amnionless (AMN) were assessed following manufacture's protocol. Briefly, 50 µL of diluted urine (1:10) was incubated in the chips overnight for immunocapture of the uEVs. After successive washing steps, a permeabilization step followed. Chips were then incubated with CF647-conjugated anti-SLC27A2 (MA5-31907, Invitrogen) and CF594-conjugated anti-AMN (HPA000817, Merck) antibodies labelled with Alexa Fluor Conjugation Kits, Fast (Abcam). Samples were analysed in triplicate and data were expressed as the mean of the three replicate spots. The number of total captured particles and urine creatinine (mg/dL) values were used for data normalization. GraphPad Prism 8 (version 8.0.2; GraphPad Software) was used for statistical analysis. The ROUT method was applied to detect outliers based on the FDR (95% confidence level), and Normality and LogNormality tests were performed to apply *t*-test (if normality was confirmed by Shapiro–Wilk test) or Mann–Whitney nonparametric test. Differences were considered significant at $p \leq 0.05$.

2.7 | Immunohistochemistry of SLC27A2 in human kidney

Kidney biopsies from hypertensive patients were obtained from Biobank of Fundación Jiménez Díaz Hospital and classified in ACR < 30 mg/g ($n = 5$) or ACR > 30 mg/g ($n = 4$). Formalin fixed paraffin embedded tissue sections were incubated overnight with primary antibody: SLC27A2 (Sigma–Aldrich, ref: HPA026089) or AMN (Sigma–Aldrich, ref: HPA000817). Negative staining controls were performed in the absence of primary antibody. Representative images for renal tissues were captured at ZEISS Axioscope 5 with Zen 3.1 Blue Edition at 10X in order to a better visualization of all the renal structure. Quantitative analysis was carried out with Image-Pro plus software (version 4.5) using 20 images per tissue at 20X. Results were expressed as positive staining per square millimetre per area. For statistical analysis, GraphPad Prism 8 software was used (version 8.0.2). *t*-test was applied, prior confirmation of normality distribution by Shapiro–Wilk test.

3 | RESULTS

Table 1 describes the baseline characteristics of the study population. No significant differences were observed in anthropometric, metabolic and blood pressure parameters between patients stratified according to their ACR (control, C [ACR < 10 mg/g] or high-normal albuminuria, HN [ACR 10–30 mg/g]) in either of the studied cohorts (discovery and confirmation). Figure 1(a) outlines the study workflow used for uEV analysis. Purified uEVs had a cup-shaped morphology (Figure 1b), an average size of 61 ± 3 nm in C patients and 60 ± 2 nm in HN patients (small uEVs) (Figure 1c), and were positive for the classical EV markers Alix, TSG-101 and Syntenin-1 (Figure 1d). The uEV populations in both clinical groups were also characterized by tetraspanin profiling. The uEVs positive for $CD63^+$ had an average size of 61 ± 16 nm in C patients and 60 ± 16 nm in HN patients. Similarly, the uEVs positive for $CD81^+$ (61 ± 18 and 61 ± 18 nm) and $CD9^+$ (61 ± 14 and 60 ± 14 nm). Most of the uEVs were $CD9^+$ (42%) and $CD63^+$ (41%) with a smaller number positive for $CD81^+$ (17%), and with a similar subpopulation distribution in both groups (Figure 1e). No significant differences were observed between groups in the total number of uEVs (Figure 1f).

The proteome of purified uEVs from the normoalbuminuric patients contained > 6200 proteins (Table S1), of which 480 proteins were significantly different between the C and HN groups ($p \leq 0.05$) (Figure 2a). In total, 216 uEV proteins were quantified by at least three peptides, with 87 proteins showing interactions (Figure 2b). A comparative analysis was performed with the urine proteome from C and HN patients (Santiago-Hernandez et al., 2021). Our previous analyses in urine evidenced inflammation, renal disease, immune system, ion transport and lipids metabolism as main functional categories altered in HN patients. uEVs revealed changes in lipid metabolism, immune system and kidney damage response as shared processes with urine. Differently, the uEV proteome reflected coagulation, glycan deregulation, energy metabolism and oxidative stress (Figure 2c), in agreement with previous data from the uEVs of patients with pathological albuminuria (ACR > 30 mg/g) (Wiśniewski et al., 2009).

To explore the utility of uEVs as kidney messengers and assign processes mediated by uEVs complementary to urine, we focused on differential uEV proteins which had been previously identified in urine but without showing variation in their urinary levels between the clinical groups (Santiago-Hernandez et al., 2021). A subset of 43 proteins composed that uEV panel (Figure 2d). Those proteins were annotated mostly to the tubule (39 of 43, compared with 20 annotated in the glomerulus). We previously showed protein and metabolites alterations in urine from HN patients, together with a significant increase in urine free fatty acids (FAs), which supports that tubular injury induced by albumin and its ligands or other stressors leads to

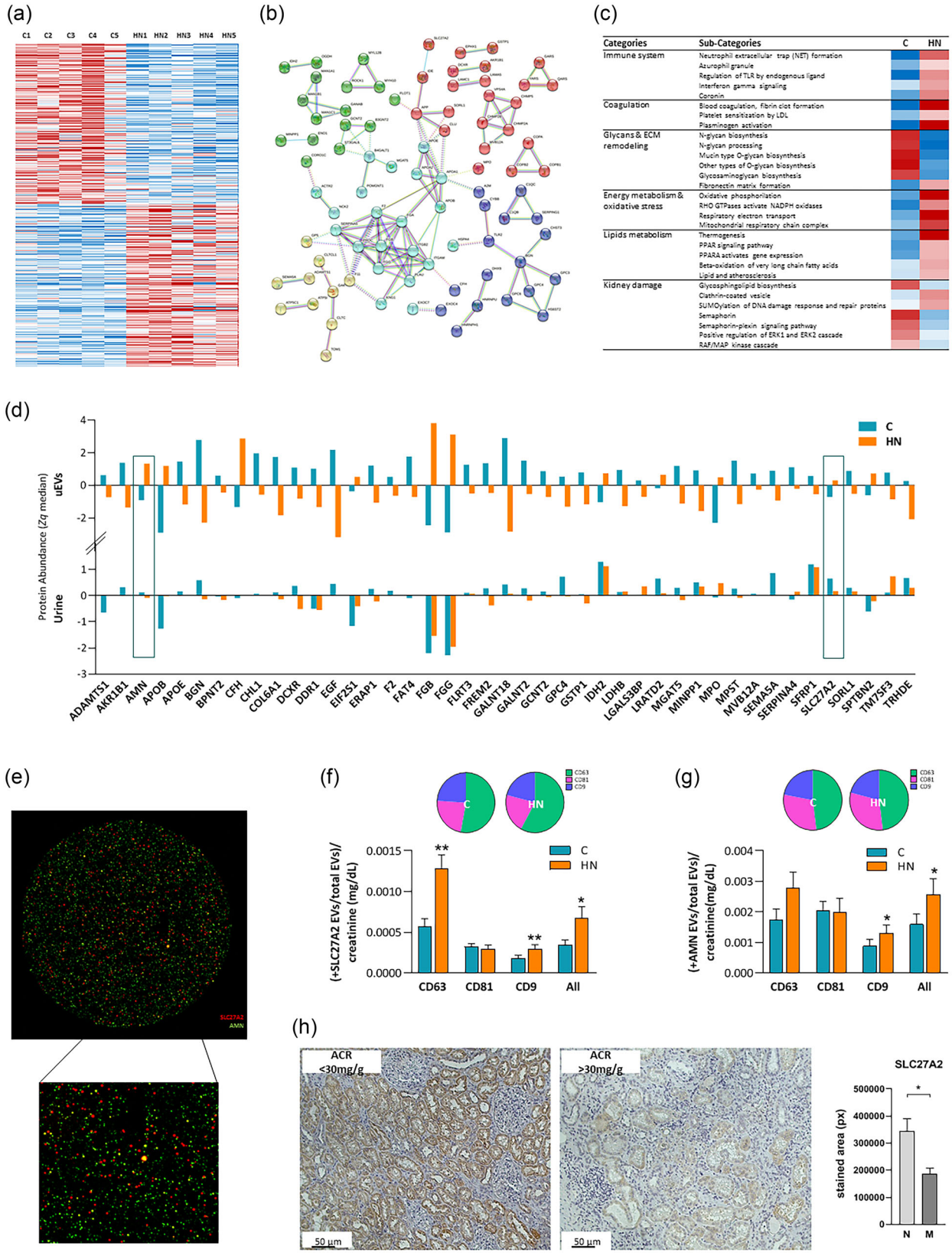

FIGURE 2 Analysis of the protein cargo of urinary extracellular vesicles reveals changes to tubular proteins that precede CKD. A subset of the protein cargo of uEVs is significantly different between C and HN patients (a), and highlights protein interaction networks (b) and altered functional categories (c) potentially associated with early renal risk (red increased; blue decreased). (d) A specific subset of 43 proteins is altered in the uEV proteome of HN patients but not in urine. The tubular proteins SCL27A2 and AMN were directly quantified by ExoView prior to uEV isolation (e) and significant increases were noted for both SCL27A2 (f) and AMN (g) confirming mass spectrometry data. Tubular SLC27A2 was quantified in biopsies from hypertensive patients with ACR < 30 mg/g versus ACR > 30 mg/g (h), evidencing reduced expression in the latter and confirming the value of uEVs as carriers of tubular damage. $^{*}p < 0.05$; $^{**}p < 0.01$.

TABLE 1 Baseline characteristics of patients classified in control (C), ACR < 10 mg/g, and high-normal (HN), ACR = 10–30 mg/g.

	Discovery cohort ($n = 10$)			Confirmation cohort ($n = 32$)		
	(TMT)-LC-MS/MS (untargeted)			ExoView (targeted)		
Characteristics	**Control (C) ($n = 5$)**	**High-normal (HN) ($n = 5$)**	***p* value**	**Control (C) ($n = 16$)**	**High-normal (HN) ($n = 16$)**	***p* value**
Age	58 ± 8	65 ± 6	0.1984	59 ± 8	64 ± 6	0.0591
Sex (% male)	100	100	>0.9999	69	75	>0.9999
BMI (Kg/m^2)	30 ± 6	30 ± 8	0.8413	31 ± 5	30 ± 6	0.5896
Cholesterol (mg/dL)	157 ± 30	168 ± 32	0.5476	179 ± 41	166 ± 30	0.5207
Triglycerides (mg/dL)	84 ± 15	92 ± 14	0.3968	108 ± 35	122 ± 60	0.8744
Cholesterol HDL (mg/dL)	54 ± 16	52 ± 7	>0.9999	53 ± 17	54 ± 16	0.5207
Cholesterol LDL (mg/dL)	86 ± 18	98 ± 30	0.6905	102 ± 33	87 ± 30	0.180
Glycemia (mg/dL)	105 ± 13	105 ± 26	0.8095	103 ± 10	107 ± 20	0.8891
Uric acid (mg/dL)	6 ± 1	7 ± 2	0.5794	6 ± 1	6 ± 2	0.3755
eGFR (mL/min/1.73 m^2)	83 ± 17	76 ± 22	0.6905	84 ± 17	85 ± 20	0.8019
ACR (mg/g)	4 ± 2	20 ± 5	0.0079	4 ± 2	24 ± 9	<0.0001
Diabetes mellitus type 2 (%)	20	20	>0.9999	19	25	>0.9999
SBP (mm Hg)	144 ± 17	140 ± 15	0.8889	140 ± 16	143 ± 14	0.4721
DBP (mm Hg)	86 ± 10	80 ± 3	0.3413	85 ± 9	83 ± 8	0.6618
Antihypertensive treatment (%)						
iECAs	40	20	>0.9999	25	19	>0.9999
ARA	60	60	>0.9999	50	69	0.4725
Diuretic	20	80	0.2063	44	50	>0.9999
Calcium channel blocker	20	60	0.5238	38	75	0.0732
α-blocker	60	0	0.1667	25	0	0.1012
β-blocker	60	20	0.5238	44	25	0.4578
Other treatments (%)						
Anticoagulant	20	0	>0.9999	6	6	>0.9999
Lipid lowering	80	20	0.2063	75	56	0.4578
Antidiabetic	0	20	>0.9999	13	13	>0.9999

Note: Discovery and confirmation cohorts are included.

Abbreviations: ACR, albumin to creatinine ratio; eGFR, estimated glomerular filtration rate; TMT, tandem mass tag labelling; LC-MS/MS, liquid chromatography and mass spectrometry in tandem.

CKD progression (Alvarez-Llamas et al., 2022). These and other evidences point to tubular reabsorption as a key process in renal damage progression and albuminuria (Dickson et al., 2014), thus we further evaluated two proteins involved in albumin reabsorption in the proximal tubule: the tubular transporter long-chain fatty acid transport protein 2 (SLC27A2 or FATP2) and the apical membrane protein AMN. To validate the untargeted mass spectrometry data of the purified uEVs, SLC27A2 and AMN were analysed in uEVs without prior isolation using single particle interferometric reflectance imaging sensor (SP-IRIS) platform/ExoView, a novel platform for direct analysis of EVs in liquid biopsy (Anfaiha-Sanchez et al., 2023) (Figure 2e). Differential analysis confirmed increased levels of SLC27A2 and AMN in uEVs from HN patients. The majority of the uEVs containing either SLC27A2 or AMN were CD63$^+$ (53% for SLC27A2 in C and 58% in HN, Figure 2f; and 48% for AMN in both groups, Figure 2g). The presence of SLC27A2 was similar in CD81$^+$ and CD9$^+$ subpopulations in both C and HN groups, whereas the distribution of AMN was slightly greater in CD81$^+$-uEVs than in CD9$^+$-uEVs for both groups. Overall, the increase in both proteins was observed mainly in CD63$^+$- and CD9$^+$-uEVs but not in CD81$^+$-uEVs. Taken together, these data identify and confirm changes in tubular proteins in subclinical albuminuria that can be directly monitored in urine through uEV analysis. To evaluate if uEVs can be used to monitor tubular damage within the kidney structure, a pilot study was performed in kidney tissue from hypertensive patients with ACR values within or out of the pathological range (ACR > 30 mg/g). SLC27A2 was localized in the tubule as expected and showed significant reduction in patients with ACR > 30 mg/g (Figure 2h).

4 | DISCUSSION

Here we show how uEVs protein cargo reflect pathophysiological alterations in subjects not meeting criteria for cardiorenal risk in daily clinical practice, as they present ACR values within normality. These findings support the need to include the measurement of ACR in routine tests and further complement it with biological indicators. We also demonstrate the complementary of uEVs to urine as biomarkers reservoir.

Our results show that subclinical albuminuria, below clinical criteria for CKD, does not affect the concentration, size or tetraspanin profile of uEVs but it does alter their protein cargo. The present findings are consistent with our previous data for patients with pathological albuminuria (ACR > 30 mg/g) (Wiśniewski et al., 2009), here demonstrating alterations in proteins and biological processes occurring in early CKD potentially mediated by uEVs. Albuminuria reflects a malfunction in both glomerular filtration and, discovered more recently, in tubular reabsorption (Anfaiha-Sanchez et al., 2024). Our results in HN patients may suggest an incipient molecular impairment of the glomerular filtration barrier in view of the reduced biosynthesis of the main components of the glomerular basement membrane: glycosaminoglycans (heparan sulphate, heparin), laminins, O-glycans and N-glycans (Figure 2c, Table S1), (Song et al., 2017; Reily et al., 2019). Neutrophil azurophilic granules were also identified as a significantly increased functional category in the HN group together with a major granule component myeloperoxidase (MPO), which regulates the formation of neutrophil extracellular traps (NETs). The increased formation of NETs promotes various kidney diseases and is evident in HN patients (Nakazawa et al., 2018). These results thus indicate an early inflammatory state even in the normoalbuminuria stage, in line with our previous findings in later stages (Gonzalez-Calero et al., 2016).

There is a strong correlation between changes in kidney and uEV cargos, particularly for tubular transporters (Rudolphi et al., 2023). We here observed an increase in the abundance of the tubular transporter SLC27A2 in uEVs from HN patients, accompanied by increased peroxisome proliferator-activated receptor α (PPARα) signalling and gene expression activation (Figure 2c). SLC27A2 is a major apical proximal tubule fatty acid (FA) transporter that regulates FA entry into tubule epithelial cells, and it has been proposed as a target for preventing CKD progression (Khan et al., 2018). During proteinuria, the hyperfiltration of albumin-bound FAs and proximal tubule FA reabsorption may cause cell death and tubular atrophy. PPARα regulates FA metabolism and activates genes encoding FA transport proteins (Kamijo et al., 2007), which is in agreement with the higher abundance of SLC27A2 observed in the HN group and with increased urinary levels of free FAs and fatty acid binding protein 1 (FABP1) previously observed (KDIGO 2012, 2012).

Impaired reabsorption was also evident by changes in the uEV levels of AMN, a membrane-bound component that, with cubilin, forms the CUBAM receptor complex, which is expressed in the apical membrane of proximal tubular epithelial cells. CUBAM is responsible for albumin reabsorption by endocytosis through clathrin-coated vesicles. Vitamin D binding-protein is also reabsorbed by proximal tubule cells through the endocytic megalin-cubilin receptor complex, which is dependent upon AMN (Gembillo et al., 2020). We found increases in clathrin-coated vesicle processes in HN patients together with a greater abundance of two constitutive proteins, clathrin heavy chain 1 (CLTC) and clathrin heavy chain 2 (CLTCL1). Levels of vitamin D binding-protein have previously been found increased in urine from HN patients and decreased in kidney tissue from patients with moderately increased albuminuria (Santiago-Hernandez et al., 2021). These data altogether support the notion that tubular malfunction is already evident in the normoalbuminuria stage.

There is no consensus marker that can be used for the robust normalization of uEV quantity and/or content (Welsh et al., 2024). In the confirmation stage, data were normalized to urinary creatinine to counterbalance data variance caused, for example, by liquid intake, as urinary creatinine is the standard reference in nephrology. Despite the use of urinary creatinine may have limitations in biomarkers data interpretation particularly in CKD, acute kidney injury and in kidney transplantation (Waikar et al., 2010) here we do not expect significant bias among patients, as all them are out of the diagnosis criteria for CKD (eGFR < 60 mL/min/1.73 m^2 or ACR > 30 mg/g). In this study, the identification of corona proteins cannot be fully discarded (Tóth et al., 2021). To mitigate such contribution, we have focused on proteins which although they were identified in urine from these patients, they did so without significant variation. For a clinical translation purpose, direct quantitation of SLC27A2 and AMN uEV proteins was performed in urine without prior centrifugation, thus partial contribution from cell remnants could not be fully discard. However, minimal influence from cell debris is expected as it had been removed by double centrifugation prior to LC-MS/MS analyses (discovery phase) and significantly different abundance was also observed for both uEV proteins. Only male patients were included in the discovery phase to minimize heterogeneity which could have had an impact considering the limited sample size. With translational purposes, the validation cohort included both male and female patients.

In conclusion, here we show the complementarity of uEVs with urine, their potential to reflect kidney damage in a subclinical condition, and the possibility to monitor tubular health by directly measured the uEVs in 5 μL urine without prior isolation. Our data indicate that alterations in the EV-mediated molecular profile are already evident before pathological ACR levels are reached. The direct quantitation of SLC27A2 and AMN in uEVs supports previous findings of defective tubular reabsorption in patients not meeting KDIGO criteria for CKD and pave the way for the use of uEVs in molecular diagnosis and monitoring of kidney disease.

AUTHOR CONTRIBUTIONS

Miriam Anfaiha-Sanchez: Investigation (lead); resources (equal); validation (lead); visualization (lead); writing—original draft (lead); writing—review and editing (lead). **Aranzazu Santiago-Hernandez**: Investigation (equal); resources (equal); visualization (equal); writing—review and editing (equal). **Juan Antonio Lopez**: Formal analysis (equal); investigation (equal); software (equal); writing—review and editing (equal). **Nerea Lago-Baameiro**: Investigation (equal); resources (equal); validation (equal); writing—review and editing (equal). **Maria Pardo**: Investigation (equal); resources (equal); validation (equal); writing—review and editing (equal). **Ariadna Martin-Blazquez**: Investigation (equal); resources (equal); writing—review and editing (equal). **Jesus Vazquez**: Funding acquisition (equal); software (equal); writing—review and editing (equal). **Gema Ruiz-Hurtado**: Conceptualization (supporting); investigation (equal); writing—review and editing (equal). **Maria G Barderas**: Conceptualization (supporting); investigation (equal); writing—review and editing (equal). **Julian Segura**: Conceptualization (equal); investigation (supporting); writing—review and editing (equal). **Luis M Ruilope**: Conceptualization (lead); investigation (supporting); writing—review and editing (equal). **Marta Martin-Lorenzo**: Conceptualization (equal); funding acquisition (equal); investigation (equal); resources (equal); supervision (lead); writing—original draft (lead). **Gloria Alvarez-Llamas**: Conceptualization (lead); funding acquisition (lead); investigation (equal); project administration (equal); supervision (lead); visualization (lead); writing—original draft (lead); writing—review and editing (equal).

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CONFLICT OF INTEREST STATEMENT

All the authors declared no competing interests.

DATA AVAILABILITY STATEMENT

The authors confirm that the data supporting the findings of this study are available within the article and its supplementary materials.

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