Acute and chronic glomerular damage is associated with reduced CD133 expression in urinary extracellular vesicles

ACUTE AND CHRONIC GLOMERULAR DAMAGE IS ASSOCIATED WITH REDUCED CD133 EXPRESSION IN URINARY EXTRACELLULAR VESICLES

Veronica Dimuccio[1], Licia Peruzzi[2], Maria Felice Brizzi[3], Enrico Cocchi[3], Fabrizio Fop[4], Alberto Boido[1], Maddalena Gili[3], Sara Gallo[3], Luigi Biancone[3,4], Giovanni Camussi[3] and Benedetta Bussolati[1*]

[1]Department of Molecular Biotechnology and Health Sciences, University of Turin; [2]Pediatric Nephrology Unit, Regina Margherita Children's Hospital, Città della Salute e della Scienza di Torino, [3]Department of Medical Sciences, University of Turin; [4]Division of Nephrology Dialysis and Transplantation, Città della Salute e della Scienza di Torino; Turin, Italy.

RUNNING HEAD: CD133+ urinary EVs in acute and chronic renal damage

***Corresponding author:** Benedetta Bussolati, Molecular Biotechnology Centre, University of Torino, via Nizza 52, 10126 Torino, Italy. Tel. 011-6706453, e-mail: benedetta.bussolati@unito.it.

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ABSTRACT

Extracellular vesicles released into urine (uEVs) can represent interesting biomarkers of renal cell damage. CD133, a stem/progenitor cell marker expressed by renal progenitor cells, is highly expressed in the uEVs of healthy subjects. We here evaluated the level of CD133 in the uEVs of patients with acute and chronic glomerular damage by cytofluorimetric analysis. The level of $CD133^+$ uEVs was significantly decreased in paediatric acute glomerulonephritis patients during the acute phase of renal damage, while it was restored after the subsequent recovery. A similar decrease was also observed in chronic glomerulonephritis patients. Moreover, $CD133^+$ uEVs significantly declined in type 2 diabetic patients, used as validation group, with lowest levels in albuminuric patients with diabetic nephropathy. Indeed, ROC curve analysis indicates the ability of $CD133^+$ uEV values to discriminate the health condition from that of glomerular disease. In parallel, a significant decrease of CD133 in renal progenitor cells and in their derived EVs was observed *in vitro* after cell treatment with a combination of glucose and albumin overload, mimicking the diabetic condition. These data indicate that the level of $CD133^+$ uEVs may represent an easily accessible marker of renal normal physiology and could provide information on the "reservoire" of regenerating cells within tubules.

INTRODUCTION

The assessment and diagnosis of renal function during acute and chronic pathologies affecting the renal compartments is, at present, mainly based on the levels of retention markers such as creatinine and urea. In addition, alternative blood and urinary biomarkers appear to be precious tools which can refer not only to the degree of renal damage but also to the pathophysiological state of the kidney. In particular, urine detection markers already used in the clinical practice (13), such as NGAL and Cystatin C, appear as the most promising ones as they directly derive from the renal compartment and from cells of the urinary tract. In addition, urine is a ready available sample easy to collect without the need of any invasive procedure.

Extracellular vesicles (EVs) are round vesicles released in biological fluids by almost all types of cells, and they contain proteins and other molecules directly correlated with the tissue from which they originate. Particularly, urinary EVs (uEVs) carry molecules that are characteristic of the epithelial cells present in the whole length of the urinary tract (23, 25). Accordingly, uEVs are attracting increasing interest as potential urinary biomarkers, since they may be a precious source of information related to the pathophysiological state of renal tissue. Indeed, data have been so far provided on the role of uEVs as biomarkers of glomerular diseases, including diabetes, tubular damage and renal fibrosis (4, 22, 27, 28).

CD133 molecule represents a marker of a population of tubular cells, intercalated between the other epithelial cells, with the ability to survive after damage and proliferate in response to cell injury, suggesting a role of this protein as marker of renal regenerative capability (3). CD133 itself may play a role in the increased proliferation of regenerating clonal tubular cells in acutely damaged kidney through the permissive effect on the activation of the Wnt/β-Catenin pathway (2). Interestingly, we previously reported that CD133, which is expressed at high level by the uEVs of healthy subjects, was reduced in the uEVs of transplanted patients with acute tubular necrosis occurring after a renal transplantation and increased in the first week following transplant (7). These results suggest that the level of $CD133^{+}$ uEVs can mirror the regenerative state of the renal tubular

compartment in healthy and in pathological conditions. However, no data are currently available on $CD133^+$ uEV level in patients with acute and chronic glomerular damage.

It is well established that glomerular damage affects the functionality of the tubular compartment, being proteinuria a main prognostic factor for interstitial fibrosis (11, 12). Indeed, tubular atrophy together with proteinuria represent the most reliable predictors of renal function in glomerular diseases (18, 30, 31).

Therefore, the aim of the present study was to verify if variation of $CD133^+$ uEVs level, previously observed in a group of acutely tubular damaged patients (7), also occurs in acute and/or persistent diseases involving the glomerular compartment. In this study, changes in $CD133^+$ uEVs level during time were assessed in paediatric patients with acute post-streptococcal glomerulonephritis, characterized by alteration of the glomerular filtration barrier, proteinuria and resolution by minimal medical approaches. In addition, two groups of chronic glomerular diseases were studied. The first group included patients with chronic glomerulonephritis (CGN) and proteinuria in the absence of renal function impairment, the second one included type 2 diabetic (T2DM) patients with different degrees of renal impairment as validation group.

In both acute and chronic glomerular diseases, the potential role of CD133 as urinary biomarker modulated during renal damage was investigated. Finally, in vitro studies were performed to evaluate whether $CD133^+$ EVs could be released by renal $CD133^+$ expressing cells. To this end, $CD133^+$ EV release was evaluated in cells exposed to hyperglycaemia and albumin overload.

METHODS

Study Groups

All patients enrolled in the present study provided informed written consent for the study. The study protocol was approved by the Bioethics Committee of the A.O.U. Città della Salute e della Scienza Hospital (Protocol number: 0021671). The study was conducted according to the principles expressed by the Declaration of Helsinki of 1975, as revised in 2013. The study group was composed by a total of 30 paediatric patients and 7 paediatric healthy control subjects. Urine was collected from 18 paediatric poststreptococcal GN patients (Table I) at diagnosis and before treatment. At time of resampling (1 year), patients were all treatment-free and experienced no relapse of proteinuria and/or haematuria after first remission. Samples were also collected from 12 CGN paediatric patients (Table I) with an active proteinuria and/or haematuria for at least 12 months. All patients were under minimum steroid treatment at the time of sample recovery without additive immunosuppressive drugs.

The validation group was composed by 47 adult type 2 diabetic patients admitted to the Clinic (HbA1c > 48 mmol/mol) and 13 adult healthy subjects age and sex matched. Table II shows patient and control clinical data.

Urine collection and EV isolation

Morning urine samples (around 100 ml) were collected in sterile containers. In parallel biochemical analysis were performed by the clinical laboratory of the A.O.U. Città della Salute e della Scienza Hospital. Urine samples were centrifuged at 3000 rpm for 15 min to remove whole cells, large membrane fragments and other debris. Protease Inhibitor (PI) Cocktail (Sigma-Aldrich, Missouri, USA; 100µl PI / 100ml urine) and NaN_3 10 mM (Sigma-Aldrich) were added immediately to the remaining supernatant. After filtration through 0.8 µm and 0.45 µm filters (Merck Millipore, Burlinghton, MA, USA), uEVs were collected from the samples through ultracentrifugation

(Beckman Coulter, OPTIMA™ L-100 K Ultracentrifuge, Rotor Type 70-Ti Brea, California, USA) at 100,000g for 1 hour at 4°C. The pellet was then resuspended in RPMI (Euroclone, Turin, Italy) + 1% DMSO (Sigma-Aldrich) and stored at -80°C until use.

EV quantification

EVs were quantified by nanoparticle tracking analysis (NTA), using the NanoSight NS300 system (NanoSight Ltd, Salisbury, UK), configured with a Blue 488 nm laser and a high sensitivity digital camera system OrcaFlash 2.8, Hamamatsu C1 1440 (NanoSight Ltd). Briefly, EVs stored in -80°C were thawed, strongly vortexed and properly diluted in physiologic solution (Fresenius Kabi Bad Homburg, Germany) previously filtered with 0.1 μm filter (Merck Millipore). For each sample, three videos of 30-second duration were recorded. The settings of acquisition and analysis were optimized and kept constant between (19).

Transmission Electron Microscopy

Transmission electron microscopy was performed using a Jeol JEM 1010 electron microscope (Jeol, Tokyo, Japan) on uEVs coated on 200 mesh nickel formvar carbon-coated grids (Electron Microscopy Science, Hatfield, PA, USA). EVs were placed in adhesion for 20 min on the grids that were then incubated with 2.5 % glutaraldehyde containing 2 % sucrose. After washings in distilled water, EVs were negatively stained with NanoVan (Nanoprobes, Yaphank, NK, USA) and analysed.

Cytofluorimetric analysis

EVs were bound to surfactant-free white aldehyde/sulfate latex beads 4% w/v, 4 μm diameter (Molecular Probes, Thermo Fisher, Waltham, MA, USA) for the subsequent cytofluorimetric analysis. Thirty μg of EVs were incubated with five μl of beads for 30 minutes at room temperature and subsequently for 30 minutes at +4°C. Adsorbed EVs were placed in different vials and

incubated with the antibodies reported in Supplementary Table I, with a final dilution of 1:50, for 15 minutes at +4°C. The adsorbed EVs were then washed and analyzed with a FACSCalibur and CellQuest software (Becton Dickinson Bioscience Pharmingen, Franklyn Lake, NJ, USA) (Supplementary Table I). The anti-Aquaporin-1 antibody (AQP1) (Santa Cruz Biotechnology, Dallas, Texas, USA) was conjugated to an Alexa Fluor® 488 dye through the Alexa Fuor® Antibody Labeling Kit (Molecular Probes) following the manufacturer's instructions. During the cytofluorimetric acquisition the gating strategy was set on the physical parameters dot plot. Controls corresponded to EVs adsorbed on beads and marked with FITC-, PE- or APC- conjugated Mouse IgG1 Isotypes (all purchased by Miltenyi, Bergisch Gladbach, Germany).

uEVs floating purification

To avoid protein contamination and to increase the purity of uEVs, a floating protocol through a sucrose gradient was applied as previously described (5, 16). Briefly, uEVs were washed with an ultracentrifuge and resuspended in 1.35 ml of buffer (0.25 M sucrose, 10 mM Tris pH 8 and 1 mM EDTA, all products purchased by Sigma-Aldrich), transferred to a SW55Ti rotor tube (Beckman Coulter) and mixed with 60% stock solution of Optiprep (Sigma-Aldrich) 1:1 ratio. Next, 1.2 ml Optiprep 20% solution was layered on top, followed by 1.1 ml of Optiprep 10% solution. The tubes were then ultracentrifuged at 350,000 g for 1 hour at +4°C with "no brake" deceleration. The next day, five fractions of 1 ml each were collected from the top of the tubes, and each fraction was diluted in 25 ml PBS (Lonza, Basel, Switzerland) and washed with an ultracentrifugation at 100,000 g for 2 hours at +4°C to pellet the EVs. The highest fraction, containing pure EVs, as assessed by NanoSight analysis, was then resuspended in 200 µl of serum free DMEM (Lonza) with 1% P/S and 1% DMSO (both products purchased by Sigma-Aldrich) to allow freezing storage in -80°C until use.

Cell culture

$CD133^+$ Renal Progenitor Cells (RPCs) were obtained from biopsies of normal tissue of a human surgically removed kidney for polar carcinoma performed (after the approval of ethical committee for the use of human tissue of Molinette Hospital; n. 168/2004), as previously described (2). In particular, the outer medullary tissue at the opposite pole of the tumor was used. The absence of tumor infiltrating cells was evaluated by pathologists. Tissue samples were cut to obtain 3–5 mm^3 fragments, digested in 0.1% collagenase type I (Sigma-Aldrich) for 30 min at 37°C and subsequently forced through a graded series of meshes for the separation of cell components from stroma and aggregates. The filtrate was then pelleted by centrifugation. To recover $CD133^+$ cells, the single cell suspension underwent magnetic separation for CD133/1 antigen (Miltenyi). $CD133^+$ cells (>98% as evaluated by cytofluorimetric analysis) were resuspended in expansion medium (Endothelial Cell Growth Basal Medium plus supplement kit, Lonza) and plated at density $1x10^4$ viable cells/cm^2.

From passages 2 to 4 cells were 48 hours treated with Human Serum Albumin (HSA) (Kedrion, Wilmington, DE, USA) at different concentrations (0.1, 1 and 10 mg/ml), Glucose (Sigma Aldrich) at different concentrations (5.5 and 27.5 mM) or a combination of HSA 10 mg/ml plus Glucose 27.5 mM. After 48 hours of treatment and overnight cell starvation, EVs were recovered by ultracentrifugation (2 hours, 100,000 g at +4°C).

Western Blot

Proteins from cells were extracted using RIPA buffer, containing 1% of Phosphatase Inhibitor Cocktails, 1% Protease Inhibitors and 1% PMSF (all purchased by Sigma-Aldrich), while proteins from EVs were obtained using the same lysis buffer on the ultracentrifuge pellet. Total protein concentration was estimated by Bradford (Bio-Rad, Hercules, CA) quantification. Ten μg of EV-lysates and 50 μg of cells lysates were loaded on Mini-PROTEAN TGX pre-cast electrophoresis gels (Bio-Rad). Proteins were subsequently transferred on iBlot nitrocellulose membranes

(Invitrogen, Carlsbad, California, US) and blotted with antibodies against CD133 (Miltenyi), Actin (Santa Cruz), HSP70/HSC70 (Santa Cruz), Rab5 (Santa Cruz) as reported in Supplementary Table I. Chemiluminescent signal was detected using the ECL substrate (Bio-Rad).

Statistical analysis

The expression of different patient data followed a non-normal distribution, as assessed by Kolmogorov-Smirnov test, therefore non parametric tests, such as Mann-Whitney or Kruskal-Wallis, were performed. Western blot experiments were analysed with student's t-test and with ordinary one-way ANOVA with Dunnett's multicomparison test. A p-value < 0.05 was considered significant.

Sensitivity and specificity of CD133 as marker of renal damage, and 95% confidence intervals (95% CI) were calculated using receiver operating characteristic (ROC) curves. The accuracy of the test among different groups of patients was done evaluating areas under curve (AUC) and p-values.

RESULTS

Characterization of uEVs from healthy paediatric subjects

EVs were isolated from urine obtained from paediatric healthy subjects using a protocol based on filtration and sequential centrifugations. Transmission electron microscopy (TEM) images showed a heterogeneous population of rounded in shape vesicles (Fig. 1A). uEV were also analysed for number and size by Nanosight (mean diameter 265.1±69nm) (Fig. 1B).

Cytofluorimetric analysis was performed using uEV adsorption on latex beads, as this method allows the analysis of small vesicles that cannot be analysed using direct cytofluorimetric analysis. The gating strategy of the evaluated beads and the setting of the negative threshold is shown in Fig 1C. The results showed that uEVs from paediatric healthy subjects, as those obtained from adult healthy subjects (7), were positive for CD24 and AQ1 renal markers, for the exosomal marker CD81 and for the progenitor/stem cell marker CD133. Urinary EVs did not express the vascular marker VEGF receptor 2 or the podocyte marker podocalyxin (PDX) (Supplementary Fig. 1A). As negative control, beads were incubated with antibodies in the absence of EVs and they did not show positive fluorescence (Supplementary Fig. 1B).

Characterization of uEVs from paediatric patients with acute and chronic GN

uEVs from four different groups of patients suffering of glomerulonephritis were compared: healthy subjects, chronic glomerulonephritis (CGN), acute post-infective glomerulonephritis (AGN), either at diagnosis (AGN T1) and on day 365, corresponding to the recovery phase (AGN T365). The Nanosight analysis showed no significant differences in the mean and mode size of the uEVs among the four groups (Fig. 2, A-B). At variance, EV count revealed that the amount of uEVs per ml of urine was significantly decreased in CGN patients compared to healthy subjects (Fig. 2C).

Cytofluorimetric analysis was performed to evaluate the relative amount of CD133 and CD81 positive uEVs, testing the same quantity of EVs previously shown to saturate the latex beads

surface (7). Indeed, as shown in Figure 3, the exosomal marker CD81 did not significantly vary among different groups, indicating an equal EVs loading. At variance, we observed a significant decrease of CD133 in uEVs of CGN patients compared to healthy subjects. Moreover, $CD133^+$ uEVs were also significantly decreased in AGN patients during the acute phase of the disease, to return to basal level on day 365. All these results are reported in dot plots graphs (Fig. 3A) and in the relative box plots (Fig. 3, C-D). These data indicate that $CD133^+$ uEVs, which mainly derived from renal tubular cells (23), are decreased after glomerular damage in both acute and chronic conditions.

Moreover, receiver operating characteristic (ROC) curves were performed, using the values determined by the cytofluorimetric analysis, to investigate the potential utility of CD133 as predictive biomarker of renal damage. By comparing healthy subjects to acute glomerulonephritis patients at day1 (AGN T1) the area under the curve (AUC) value for CD133 was 0.878, with an OR (CI 95%) of 19.5 (3-126.5) and $p = 0.001$ (Fig. 3D). In the context of this analysis the percentage of $CD133^+$ uEVs considered as threshold able to discriminate healthy subjects and acutely damaged glomerulonephritis patients resulted 36.

Characterization of uEVs from diabetic patients

We subsequently aimed to validate the reduction of $CD133^+$ uEVs observed after glomerular damage in paediatric patients, in patients affected by T2DM-associated glomerular disease. Based on the degree of their renal impairment, patients were divided in three groups: normoalbuminuric diabetic patients (NAlb DN), microalbuminuric diabetic patients (MiAlb DN) and macroalbuminuric diabetic patients (MaAlb DN), and were compared to age and sex matched healthy subjects. Nanosight analysis showed that the mean and mode uEV dimension are similar among groups. Similarly, no differences in the number of uEVs /ml of urine were detected (Fig. 2, D-F).

The presence of $CD133^+$ uEVs in T2DM patients was subsequently compared to that of healthy subjects by cytofluorimetric analysis. In Figure 4B-C the level of CD81 did not change between healthy and diabetic patients, while $CD133^+$ uEVs was significantly decreased in DN patients. Moreover, when the analysis of $CD133^+$ uEVs was based on their level of albuminuria, a further decrease of $CD133^+$ uEVs was detected in macroalbuminuric patients (Fig. 4D).

In order to evaluate whether proteins in the urine of albuminuric T2DM patients could affect the results of the cytofluorimetric analysis, three uEV pools belonging to the three different T2DM groups were purified from contaminating proteins by applying a glucose gradient floating protocol (5, 16). Floated uEVs were analysed and compared to a pool of healthy uEVs subjected to the same protocol. As reported in Figure 4E the loading of 10 micrograms of proteins from each sample showed a significant decrease in CD133 in all T2DM patients, confirming the results of the cytofluorimetric analysis.

ROC curves were also performed on the results obtained from the cytofluorimetric analysis, in order to confirm CD133 as a potential predictive biomarker of renal damage. By comparing healthy subjects to all T2DM patients the AUC value for CD133 was 0.775, with an OR (CI 95%) of 9.8 (2.2-42.7) and $p = 0.001$ (Fig. 3D). In the context of this analysis the percentage of $CD133^+$ uEVs considered as threshold able to discriminate healthy subjects and diabetic patients resulted to be 37.

Modulation of $CD133^+$ EV release by $CD133^+$ cells subjected to Albumin and Glucose overload

To evaluate the contribution of renal progenitor cells in the release of $CD133^+$ uEVs , $CD133^+$ renal cells were treated with different concentrations of albumin and glucose for 48h (Fig. 5A). As shown in Fig 5B, albumin (0.1-10 mg/ml) but not glucose (5.5-27.5 mM) treatment induced a dose dependent loss of CD133 expression by $CD133^+$ cells (Fig. 5B). Likewise, albumin load induced the loss of $CD133^+$ in $CD133^+$ renal cell derived EVs (Fig. 5C). To mimic diabetes *in vitro*, $CD133^+$ cells were treated with high albumin concentration (10 mg/ml) together with high glucose

concentration (27.5 mM), for 48 hours. Western blot revealed a strong decrease in CD133 expression both in progenitor renal cells and their derived EVs (Fig. 5, D-E). These data support the results obtained by analysing patients' urine, and indicate that proteinuria and glucose may modify tubular progenitors cells and promote CD133 loss in cells and in their derived uEVs .

DISCUSSION

EVs are constantly released by virtually all cells and their characteristics reflect the state of the cell of origin, so that they can mirror tissue health and/or disease. In the present study, we show that the levels of $CD133^+$ uEVs, differently from normal subjects, declined in the urine of paediatric and adult patients with glomerular disease, suggesting that $CD133^+$ uEVs may represent a marker of renal normal physiology. Indeed, ROC curve analysis indicates the ability of $CD133^+$ uEV values to discriminate the health condition from that of glomerular disease.

Since uEVs released by renal tissue could be detected in human urine, EVs may be exploited as markers for the physiological/pathological state of the renal tissue. Indeed, proteomic analysis of uEVs from healthy subject demonstrated the presence of renal proteins, such as uromodulin, aquaporin 1 and aquaporin 2, indicating that the majority of EVs derived from tubular segments (9). During glomerular damage, podocyte-released EVs may be detected within urine, (17) and podocyte-derived proteins within urinary exosomes were reported as specific markers of glomerular injury and chronic renal damage. In particular, exosomal WT1 was described as a marker of focal segmental glomerulosclerosis in murine and human studies (29) while CD2AP mRNA was reported as a marker of chronic kidney disease (20). The nature of urine vesicles is heterogeneous in type and dimension, including larger vesicles, so called microparticles. Indeed, the number of podocyte-derived microparticles by itself was increased in type 1 diabetic patients, and could predict early renal damage (21).

Besides being considered as biomarkers, EVs may also play a role in the cell-to-cell communication along the urinary lumen. Indeed, proximal tubule derived EVs were shown to be internalized by distal tubular cells (8). Furthermore, the increase of water flow in recipient cells proved the functional transfer of AQP2 from EVs deriving from collecting ducts (26). During glomerular disease such as diabetes, podocyte-derived EVs may promote fibrosis of tubular cells, via

detrimental glomerular-tubular signals (24). These data suggest that uEVs may orchestrate the trafficking of different renal messages occurring during physiological or pathologic conditions.

In the present study we focused on CD133, a tubular EV marker, previously shown to be highly abundant in normal urine (7). Indeed, $CD133^+$ uEV sorting clearly showed the co-expression of proximal tubular markers (mainly Aminopeptidase and Aquaporin 1) indicating its origin from cells of the proximal tubules (7). Moreover, we herein confirmed that $CD133^+$ EVs are released, at least in vitro, by renal $CD133^+$ cells. Similarly $CD133^+$ EVs have been detected in the spinocerebral fluids in association with pathological conditions (14, 15) and in cultures of $CD133^+$ stem/progenitor cells of nervous and hematopoietic origins (1, 6). Therefore, it could be speculated that levels of urinary $CD133^+$ EVs may reflect the number of $CD133^+$ cells lining the lumen of renal tubules.

Interestingly, in paediatric patients with acute post-infective glomerulonephritis, $CD133^+$ uEVs were downregulated during the acute phase to return to basal level after 1 year, as the result of tissue repair. Similarly, $CD133^+$ uEV level was significantly low in paediatric patients with chronic renal disease. This observation suggests that CD133 could be considered a general marker of renal tissue damage that may reflect the state of the functional tubular compartment. This is consistent with data previously demonstrated in transplanted patients with slow graft function (7). Our results were further validated in diabetic patients. Indeed, we found that the reduction of CD133 levels in diabetic patients was even more evident in the subgroup of macro-albuminuric patients.

These data suggest that reduction of $CD133^+$ uEVs in patients with acute and chronic glomerular damage reflects the tubular damage occurring in the tubular compartment as a consequence of glomerular injury and protein overload. Consistently, *in vitro* experiments showed that the albumin overload decreased the expression of CD133 in cultured cells as well as in their derived EVs. Indeed, it is well established that proteinuria may accelerate kidney disease progression (10).

The analysis of ROC curves confirmed the ability of $CD133^+$ uEV levels to discriminate between healthy subjects and patients with renal disease. Moreover, the restoration of $CD133^+$ uEV levels in

patients one year after recovery from glomerular damage further supports the correlation of this marker with the renal pathophysiological condition. Considering the role of CD133 cells in renal repair and regeneration, it can be also inferred, that $CD133^+$ uEVs may represent an easily accessible marker of renal regeneration and of the "reservoire" of regenerating cells within tubules.

In conclusion, it can be speculated that the assessment of $CD133^+$ uEVs level in urine might provide a number of benefits. The use of a surface marker may allow a direct and quick evaluation compared to intra-vesicle protein, mRNA or microRNAs evaluation. Moreover, this assessment, being a percentage of total events, is independent from the number of total EVs in urine and from their concentration. Finally, the high level of $CD133^+$ uEVs in normal subjects guarantees a very good discrimination between healthy and patients. This marker may therefore provide information on the renal parenchyma status and chances associated with tissue recovery after injury without invasive procedures, which represents an important step toward biopsy overcoming and patient's safety. The limit of our study relies on the use of a semi-quantitative cytofluorimetric analysis. In fact, the absorption onto latex beads was an instrumental requirement for the assessment of small EVs. However, it should be considered that several new cytofluorimetric techniques, which are currently under evaluation, would allow a direct assessment of the small EV population. This implies that future studies may benefit of an easier and direct measurement of uEV number and markers.

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Brizzi: study design, data collection of diabetic patients and manuscript writing; Enrico Cocchi: data analysis of paediatric patients and interpretation; Fabrizio Fop: statistical analysis; Alberto Boido: data collection and cytofluorimetric analysis; Maddalena Gili: data collection of diabetic patients; Sara Gallo: data collection of diabetic patients; Luigi Biancone: study design, data interpretation; Giovanni Camussi: manuscript writing; Benedetta Bussolati: conception and design, financial support, data analysis and interpretation, manuscript writing. All authors approved the final draft.

Conflict of interest: GC is component of the Unicyte Scientific Board. VD, GC and BB are inventors in a related patent application.

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Supplemental Material can be located at:

https://figshare.com/s/cfe77926a2b726032004

FIGURE LEGENDS

Figure 1. Characterization of uEVs in healthy subjects. (A) Representative Transmission Electron Microscopy (TEM) image of uEVs isolated from healthy subjects (scale bar 150nm), and an enlargement on one single uEV (scale bar 20nm). **(B)** Nanoparticle Tracking Analysis (NTA) of a representative pool of uEVs showing their size distribution profile. Cytofluorimetric analysis of surface markers expressed by healthy subjects uEVs were represented as **(C)** dot plot (the gating strategy and the negative fluorescence controls are reported) and **(D)** histograms with isotype control (black line) plotted on the marker signal (red line). Ten different preparations were tested with similar results.

Figure 2. Characterization of uEVs recovered from patients with Acute and Chronic glomerular damage. (A, B) Representative Nanoparticle Tracking Analysis (NTA) of uEVs from each group of paediatric patients and box plot representation of Mean and Mode values of EVs size (nm). **(C)** Evaluation of EVs concentration in urine of the different groups of paediatric patients. **(D, E)** Representative Nanoparticle Tracking Analysis (NTA) of uEVs from each group of T2DM patients and box plot representation of Mean and Mode values of EVs size (nm). **(F)** Evaluation of EVs concentration in the urine of the different groups of T2DM patients. Data are expressed as mean ± SE. (*) $p<0.05$ with unpaired t-test.

Figure 3. Characterization of uEVs obtained from acute damaged patients. (A) Dot plot cytofluorimetric analysis of surface markers in uEVs derived from healthy subjects and paediatric patients. The gating strategy and the negative fluorescence controls are also reported. Box plot representation of cytofluorimetric values of CD81 (grey plot) and CD133 (red plot) percentage **(B)** comparison between healthy subjects (h) and chronic glomerulonephritic patients (CGN), or **(C)** between healthy subjects (h) and acute glomerulonephritic patients (AGN) at day 1 (T1) and year 1

(T365) after the damage. **(D)** ROC analysis for the detection of renal damage in different groups of acutely damaged patients using CD133. A table with the CD133 threshold value, the AUC value, the p-value and the odd ratios with confidence interval is also reported.

Figure 4. Characterization of uEVs recovered from patients with chronic damage. (A) Representative Nanoparticle Tracking Analysis (NTA) of uEVs recovered from healthy subjects (h) and diabetic patients (DN). Cytofluorimetric results are reported as dot plot **(C)** and box plot **(B)** of CD81 (grey plot) and CD133 (red plot) in healthy (h) and diabetic patients (DN). **(D)** Cytofluorimetric evaluation of CD133 in uEVs from healthy (h), diabetic normoalbuminuric (NAlb), diabetic microalbuminuric (MiAlb) and diabetic macroalbuminuric (MaAlb) patients. (****) $p<0.0001$, with Kruskal-Wallis multiple comparison test. **(E)** Western blot analysis of CD133 in floating purified uEVs. **(F)** ROC analysis for the detection of renal damage in different groups of diabetic patients using CD133. A table with the CD133 threshold value, the AUC value, the p-value and the odd ratios with confidence interval is also reported.

Figure 5. Decrease of CD133 upon HSA and Glucose treatment. (A) Timetable of the experiment performed on $CD133^+$ renal cells. **(B and D)** CD133 levels in $CD133^+$ cells upon treatment with increasing concentration of human serum albumin (HSA) (0.1 – 1 – 10 mg/ml) or Glucose (5.5 – 27.5 mM) (B) or upon HSA 10 mg/ml and Glucose 27.5 mM combo treatment **(D). (C and E)** CD133 levels in $CD133^+$ cells derived EVs upon HSA treatment (10 mg/ml) (C) or upon HSA 10 mg/ml and Glucose 27.5 mM combo treatment. Data are expressed as mean ± SE. (*) $p<0.05$, with unpaired t-test.

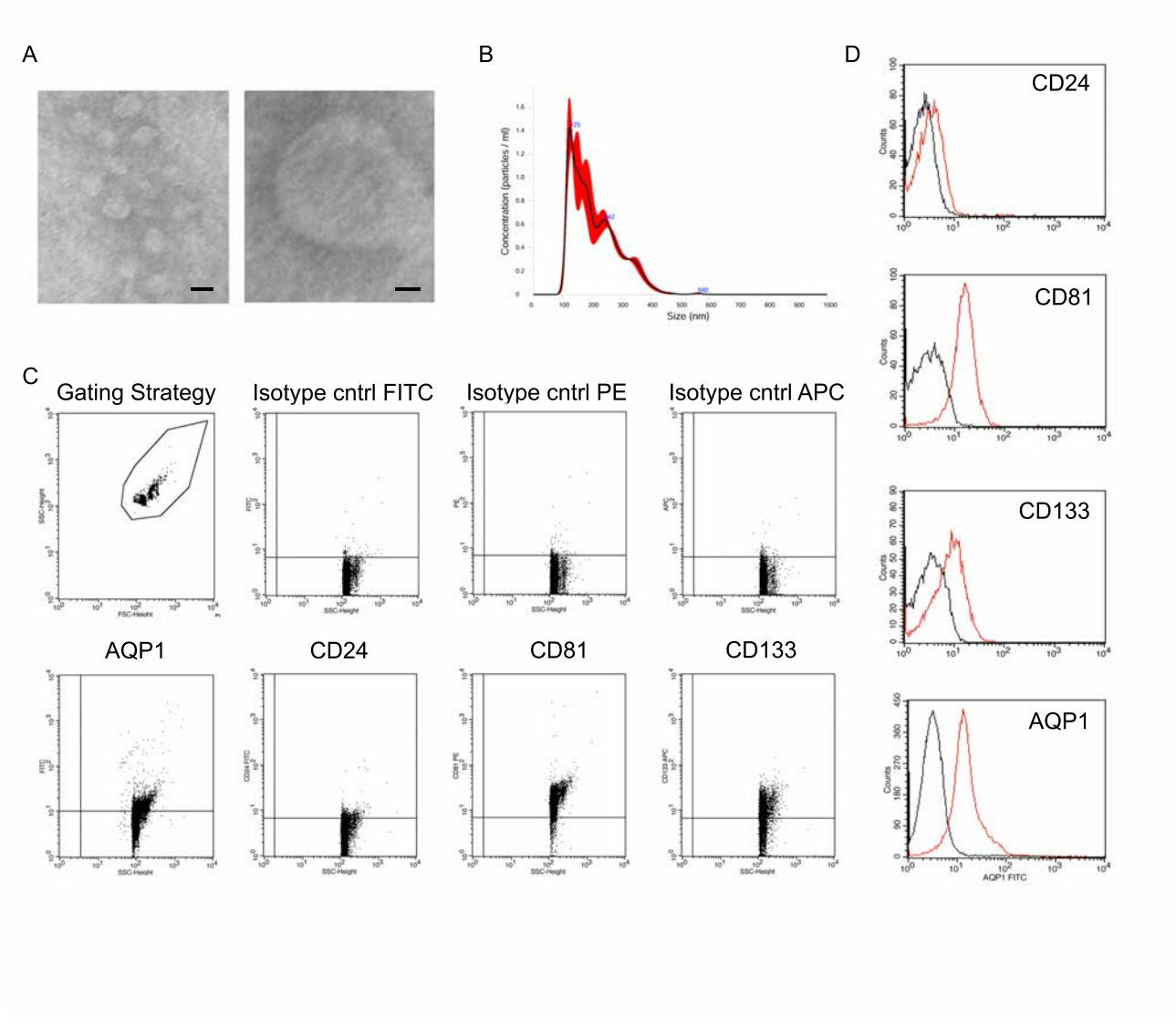

A
B
D
CD24
CD81
CD133
AQP1
Size (nm)
C
Gating Strategy
Isotype cntrl FITC
Isotype cntrl PE
Isotype cntrl APC
AQP1
CD24
CD81
CD133

Figure 1

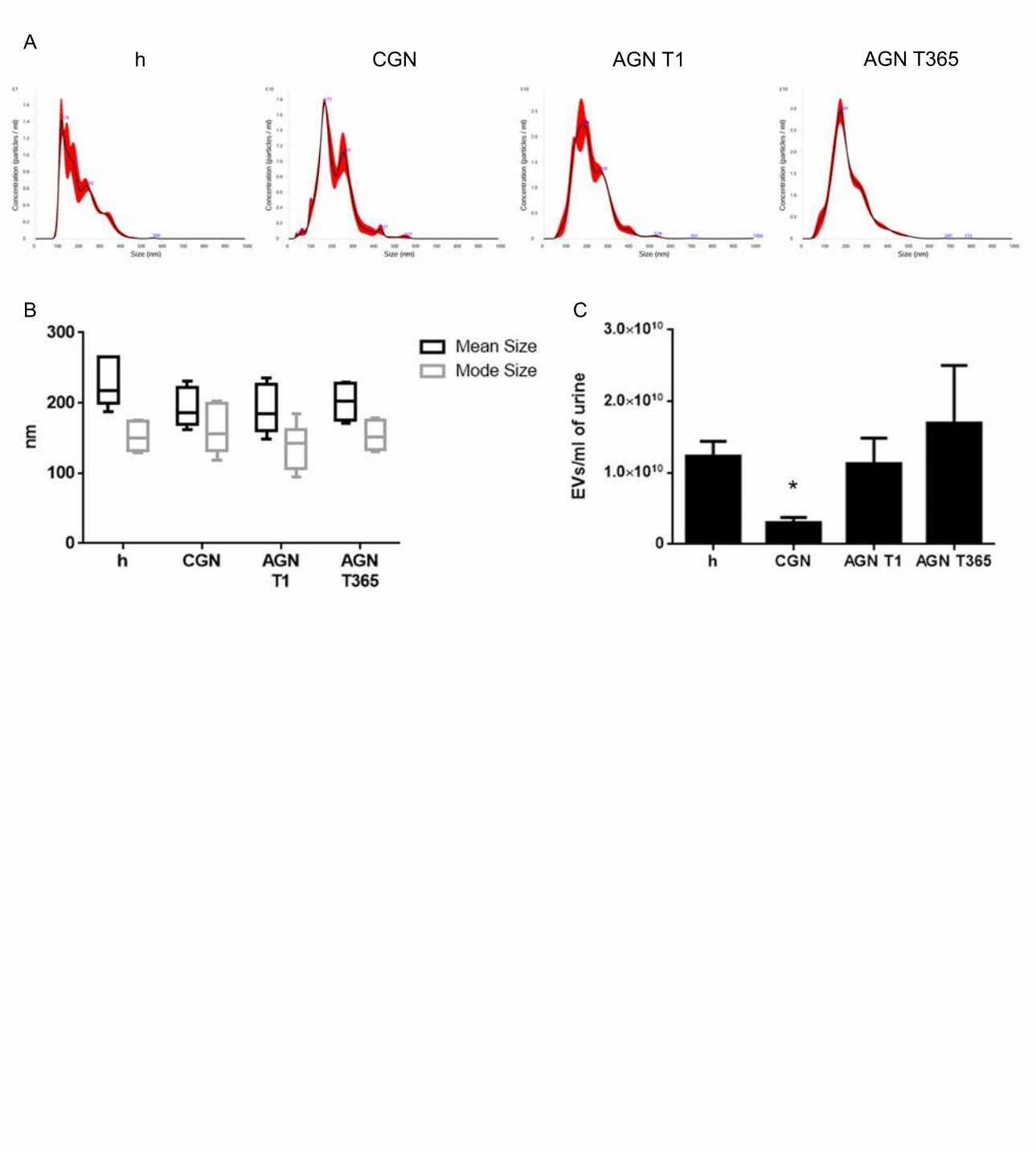
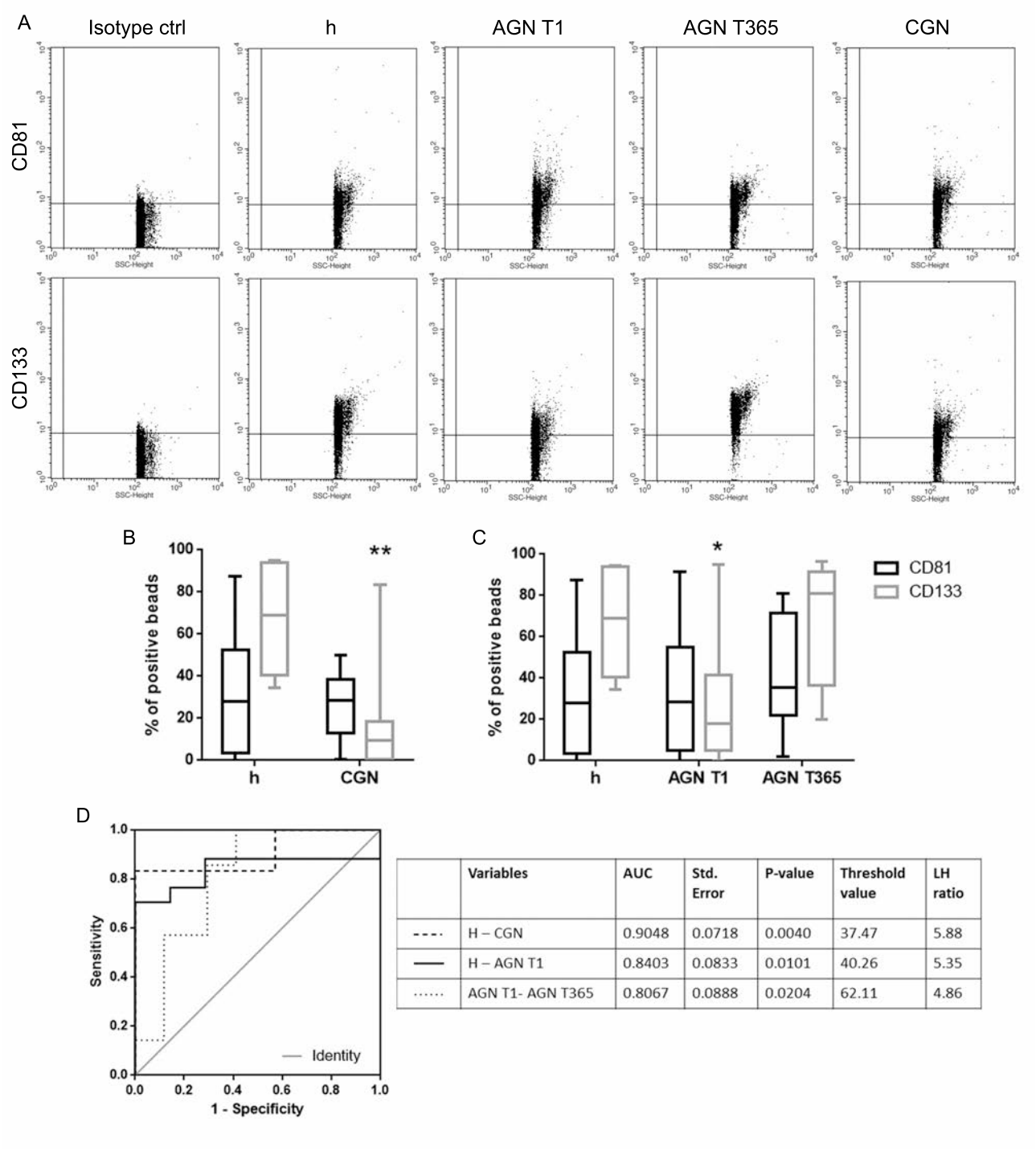
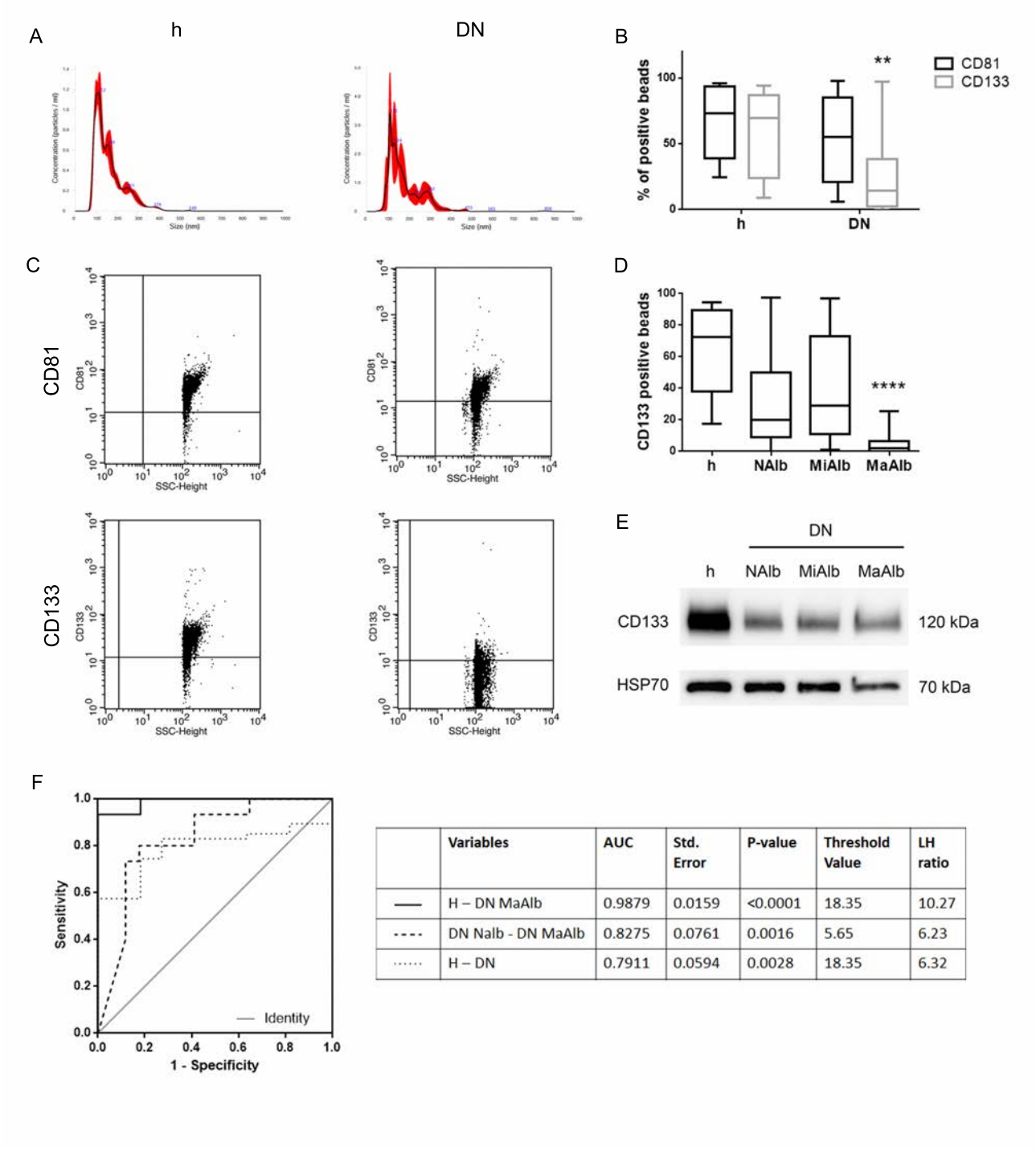

A
h
CGN
AGN T1
AGN T365
B
300
200
100
0
nm
Mean Size
Mode Size
h
CGN
AGN T1
AGN T365
C
3.0×10¹⁰
2.0×10¹⁰
1.0×10¹⁰
0
EVs/ml of urine
*
h
CGN
AGN T1
AGN T365

Figure 2



	Variables	AUC	Std. Error	P-value	Threshold value	LH ratio
- - - -	H – CGN	0.9048	0.0718	0.0040	37.47	5.88
———	H – AGN T1	0.8403	0.0833	0.0101	40.26	5.35
.....	AGN T1- AGN T365	0.8067	0.0888	0.0204	62.11	4.86

Figure 3



	Variables	AUC	Std. Error	P-value	Threshold Value	LH ratio
——	H – DN MaAlb	0.9879	0.0159	<0.0001	18.35	10.27
- - - -	DN Nalb - DN MaAlb	0.8275	0.0761	0.0016	5.65	6.23
·····	H – DN	0.7911	0.0594	0.0028	18.35	6.32

Figure 4

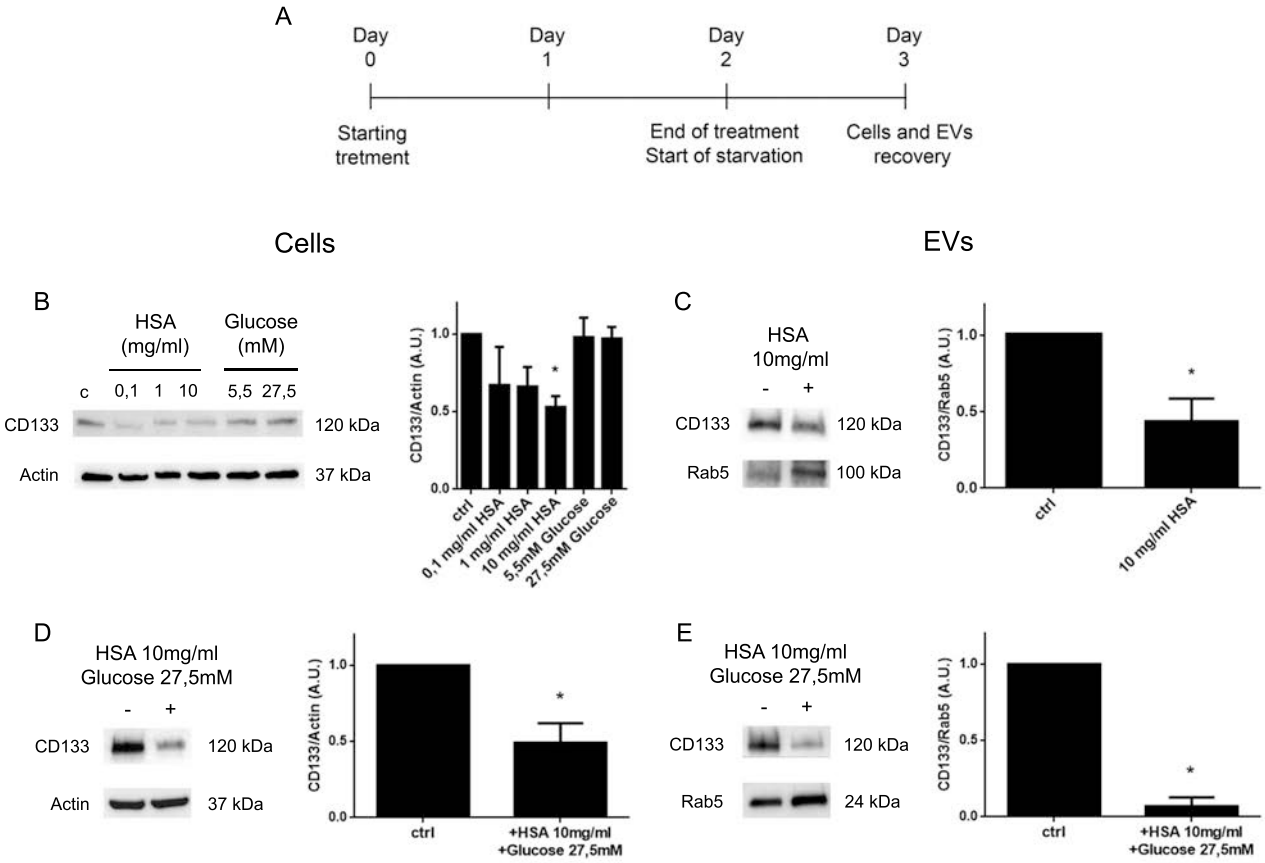

A
Day 0
Day 1
Day 2
Day 3
Starting tretment
End of treatment
Start of starvation
Cells and EVs recovery
Cells
EVs
B
HSA (mg/ml)
Glucose (mM)
c
0,1
1
10
5,5
27,5
CD133
120 kDa
Actin
37 kDa
CD133/Actin (A.U.)
ctrl
0,1 mg/ml HSA
1 mg/ml HSA
10 mg/ml HSA
5,5mM Glucose
27,5mM Glucose
C
HSA 10mg/ml
CD133
120 kDa
Rab5
100 kDa
CD133/Rab5 (A.U.)
10 mg/ml HSA
D
HSA 10mg/ml
Glucose 27,5mM
CD133
120 kDa
Actin
37 kDa
+HSA 10mg/ml +Glucose 27,5mM
E
HSA 10mg/ml
Glucose 27,5mM
CD133
120 kDa
Rab5
24 kDa

Figure 5

Groups of Patients	n.	Sex (M/F)	Median Age (y.o.)	Clinical Features	Haematuria degree	Timing of urine collection	Serum Creatinine (mg/dL)	UPCR (mg/mg)	n. EVs (EVs/ml of urine)
h	7	3/4	12 (9-13)	-	-		0.52 (±0.06)	0.13 (±0.01)	1.24E+10 (±2.05E+09)
CGN	12	11/1	9 (5-11)	Alports' Syndrome (n=2), Schonlein-Henoch (n=5), LES (n=2), Nefrosic Syndrome (n=3)	+/-		0.49 (±0.07)	0.40 (±0.12)	3.04E+09 (±7.03E+08)
AGN in Acute Phase	18	12/6	5 (4-6)	Post-Streptococcal GlomeruloNephritis	+++	At time of diagnosis	0.74 (±0.12)	0.27 (±0.06)	1.13E+10 (±3.54E+09)
AGN in Recovery Phase	8	6/2	5 (4-6)	Post-Streptococcal GlomeruloNephritis	-	One year after diagnosis	0.43 (±0.07)	0.04 (±0.02)	1.70E+10 (±8.07E+09)

Table I. Clinical characteristics of patients with chronic and acute glomerular nephropathy. Data are reported as mean ± SE. UPCR = Urinary Protein on Creatinine Ratio. Age is reported as Median (Q1-Q3).

Groups of Patients	n.	Sex (M/F)	Median Age (y.o.)	Treatment (Sartans+ACE-Inhib/others)	Serum Creatinine (mg/dL)	eGFR (ml/min/1.73m^2)	Albuminuria (mg/dL)	HbA1c (mmol/mol)	n. EVs (EVs/ml of urine)
h	13	6/7	60 (42-71)		0.79 (±0.05)	96.1 (±4.4)	0 (±0.0)	0 (±0)	3.45E+09 (±6.65E+08)
Diab NaIb	17	11/6	68 (63-77)	7/10	1.16 (±0.12)	72.4 (±6.6)	0.1 (±0.1)	74.1 (±6.2)	1.09E+10 (±5.28E+09)
Diab MiAlb	15	10/5	79 (72-85)	7/8	1.40 (±0.24)	62.4 (±6.4)	22.4 (±2.2)	58.7 (±4.4)	8.83E+09 (±1.74E+09)
Diab MaAlb	15	9/6	82 (70-87)	6/9	1.57 (±0.22)	45.1 (±7.1)	129.5 (±27.2)	58.8 (±3.1)	1.95E+10 (±1.07E+10)

Table II. Clinical characteristics of diabetic patients. Data are reported as mean ± SE. Age is reported as Median (Q1-Q3).